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Metabolic Interactions of Hormonal Steroids and Chlorinated Hydrocarbons: Effects of Neonatal Treatment with o,p'-DDT on the Development of the Steroidogenic Endocrine System of the Male Rat

Michigan Univ., Ann Arbor. Medical School

Prepared for

Health Effects Research Lab., Research Triangle Park, N.C.

Sep 77

600/1-77-042
September 1977

PB 272 669

Environmental Health Effects Research Series

METABOLIC INTERACTIONS OF HORMONAL

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TECHNICAL REPORT DATA
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PB272669

1. REPORT NO.
EPA-600/1-77-042

2.

3. RECIPIENT'S ACCESSION NO.

4. TITLE AND SUBTITLE
METABOLIC INTERACTIONS OF HORMONAL STEROIDS AND CHLORINATED HYDROCARBONS - Effects of Neonatal Treatment with o,p'-DDT on the Development of the Steroidogenic Endocrine System of the Male Rat

5. REPORT DATE
September 1977

6. PERFORMING ORGANIZATION CODE

7. AUTHOR(S)
Kenneth Lyle Campbell

8. PERFORMING ORGANIZATION REPORT NO.

9. PERFORMING ORGANIZATION NAME AND ADDRESS
The University of Michigan Medical School
Ann Arbor, Michigan 48104

10. PROGRAM ELEMENT NO.
1EA615

11. CONTRACT/GRANT NO.
R-800637

12. SPONSORING AGENCY NAME AND ADDRESS
Health Effects Research Laboratory RTP,NC
Office of Research and Development
U.S. Environmental Protection Agency
Research Triangle Park, N.C. 27711

13. TYPE OF REPORT AND PERIOD COVERED

14. SPONSORING AGENCY CODE
EPA-600/11

15. SUPPLEMENTARY NOTES

16. ABSTRACT
This study was an attempt to generate approaches to the measurements of the potential of xenobiotics to alter reproductive capacity and viability which may allow improvement of those currently in use. More specifically, it was an attempt to identify the effects of neonatal exposure to the model compound, o,p'-DDT (1,1,-trichloro-2-(2-chlorophenyl)-2-(4-chlorophenyl)-ethane), on the developing steroidogenic endocrine system of the model animal, the male rat. The effect of exposures by direct injections before day 5 of age and by indirect treatment via an o,p'-DDT injected dam were examined initially by measurement of growth, organ weights, organ histology, and of serum corticosterone and luteinizing hormone (LH). Later experiments included determinations of the effects of o,p'-DDT on the serum LH responses to challenges of the hypothalamo-pituitary axis with adult castration and repeated injections of luteinizing hormone releasing hormone (LHRH). The observed effects indicated a precocious development of the adrenal cortex in immature animals treated neonatally with o,p'-DDT similar to that seen in rats neonatally injected with 17 -estradiol-17-valerinate. They also demonstrated altered serum LH responses in treated rats to challenges with adult castration or repeated injections of LHRH. These results are discussed in relation to previous work on steroids and o,p'-DDT in several animal systems, to possible mechanisms, to prospective experiments and to the original goal of the research.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
Chlorohydrocarbons Steroids Endocrine glands Metabolism Biochemistry Rats DDT		06 A, F

18. DISTRIBUTION STATEMENT RELEASE TO PUBLIC	19. SECURITY CLASS *(This Report)* UNCLASSIFIED	21. NO. OF PAGES 324
	20. SECURITY CLASS *(This page)* UNCLASSIFIED	22. PRICE A14 - A01

EPA Form 2220-1 (9-73)

EPA-600/1-77-042
September 1977

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Effects of Neonatal Treatment with o,p'-DDT on the Development of the Steroidogenic Endocrine System of the Male Rat

by

Kenneth Lyle Campbell
The University of Michigan Medical School
Ann Arbor, Michigan 48104

Principal Investigator

Merle Mason, Ph.D.
Associate Professor of Biological Chemistry
The University of Michigan Medical School
Ann Arbor, Michigan 48104

Grant Number R-800637

Project Officer
Laurence Rosenstein, Ph.D.
Environmental Toxicology Division
Health Effects Research Laboratory
Research Triangle Park, N.C. 27711

U.S. ENVIRONMENTAL PROTECTION AGENCY
OFFICE OF RESEARCH AND DEVELOPMENT
HEALTH EFFECTS RESEARCH LABORATORY
RESEARCH TRIANGLE PARK, N.C. 27711

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FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The Health Effects Research Laboratory, Research Triangle Park, conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, non-ionizing radiation, environmental carcinogenesis and the toxicology of pesticides as well as other chemical pollutants. The Laboratory develops and revises air quality criteria documents on pollutants for which national ambient air quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is preparing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of health care and surveillance of persons having suffered imminent and substantial endangerment of their health.

The study described herein was initiated to meet program requirements concerning the improvement of in vivo measurements involved in determining the potential toxicity of xenobiotics on reproduction. The information gained from this study has permitted the evaluation of the effects on the developing endocrine system of a rat following neonatal exposure to a model compound, o,p'-DDT. It is anticipated that the model developed in this study can be applied to other compounds and species and thus serve as a useful adjunct to toxicology research.

JHKnelson

John H. Knelson, M.D.
Director,
Health Effects Research Laboratory

ACKNOWLEDGEMENTS

This project was part of the doctoral dissertation research of Kenneth Lyle Campbell, under the mentorship of Dr. Merle Mason. Support of Mr. Campbell during the tenure of his program was derived from fellowships from The University of Michigan Institute for Environmental Quality and the Albert Euclid Hinsdale Memorial Fellowship Fund and from a traineeship from the National Institutes of Health (Grant No. 5-T01-GM-00187-15) and is gratefully acknowledged.

Experimental assistance and help in the interpretation of results was obtained from the Reproductive Endocrinology Program, The University of Michigan. Dr. Fred Karsch of that program was especially helpful in interpreting the radioimmunoassays and in designing some experiments.

Maija Mitzens provided invaluable support and assistance in writing the MetFLX program and in many other aspects of research and writing.

This document is submitted as a final report under U.S. Environmental Protection Grant No. R-800637 with Dr. Merle Mason.

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LIST OF ABBREVIATIONS AND COMMON NAMES

ACTH	adrenocorticotrophic hormone
Aldrin	1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a-hexahydro-exo-1,4-endo-5,8-dimethanonaphthalene
Δ^4-	Δ^4-androstenedione; 4-androsten-3,17-dione
ANOVA	analysis of variance
BSA	bovine serum albumin
C	control
cAMP	cyclic 3',5'-adenosine monophosphate
Cholesterol	5-cholesten-3α-ol
CBG	corticosteroid binding globulin
Ci	Curie
CPE	5-cholesten-3α-ol-3-propyl ether
CRH	corticotrophin releasing hormone
DDA, DDE, DDD, DDT	either the o,p'- or p,p'-isomer
o,p'-DDA	2-(2-chlorophenyl)-2-(4-chlorophenyl)-acetic acid
p,p'-DDA	2,2-bis-(4-chlorophenyl)-acetic acid
o,p'-DDD	2,2-dichloro-1-(2-chlorophenyl)-1-(4-chlorophenyl)-ethane; 1,1-dichloro-2-(2-chlorophenyl)-2-(4-chlorophenyl)-ethane
p,p'-DDD	2,2-dichloro-1,1-bis-(4-chlorophenyl)-ethane; 1,1-dichloro-2,2-bis(4-chlorophenyl)-ethane
o,p'-DDE	2,2-dichloro-1-(2-chlorophenyl)-1-(4-chlorophenyl)-ethylene; 1,1-dichloro-2-(2-chlorophenyl)-2-(4-chlorophenyl)-ethylene

LIST OF ABBREVIATIONS AND COMMON NAMES (Cont.)

p,p'-DDE	2,2-dichloro-1,1-bis-(4-chlorophenyl)-ethylene; 2,2-bis-(4-chlorophenyl)-1,1-dichloroethylene
p,p'-DDMU	2-chloro-1,1-bis(4-chlorophenyl)-ethylene
o,p'-DDT	1,1,1-trichloro-2-(2-chlorophenyl)-2-(4-chlorophenyl)-ethane
p,p'-DDT	1,1,1-trichloro-2,2-bis-(4-chlorophenyl)-ethane
DHA	dehydroepiandrosterone; 5-androsten-3α-ol-17-one
DMSO	dimethyl sulfoxide
dpm	disintegrations per minute
EST	Eastern standard time
estradiol	17β-estradiol; 1,3,5(10)-estratriene-3,17β-diol
estriol	1,3,5(10)-estratriene-3,16α,17β-triol
EV	17β-estadiol-17-valerinate
FID	flame ionization detector
FSH	follicle stimulating hormone
GC	gas chromatography
GnRH	gonadotrophin releasing hormone
G-6-P	glucose-6-phosphate
G-6-PDH	glucose-6-phosphate dehydrogenase
hCG	human chorionic gonadotrophin
17α	17α-hydroxyprogesterone; 4-pregnen-17α-ol-3,20-dione
ICSH	interstitial cell stimulating hormone (LH)
i.p.	intraperitoneal
LD_{50}	lethal dose to 50%

LIST OF ABBREVIATIONS AND COMMON NAMES (Cont.)

LH	luteinizing hormone
LHRH	luteinizing hormone releasing hormone
Mirex	dodecachlorooctahydro-1,3,4-metheno-2H-cyclobuta [cd] pentalen-2-one
MTS	Michigan Terminal Systems
NADH	reduced nicotinamide adenine dinucleotide
$NADP^+$	nicotinamide adenine dinucleotide phosphate
OAAD	ovarian ascorbic acid depletion
oLH	ovine luteinizing hormone
P	progesterone; 4-pregnen-3,20-dione
PB	phenobarbital
PBS	phosphate-buffered-saline
PCB's	polychlorinated biphenyls
pg	picogram
POPOP	1,4-bis-[2-(5-phenyloxazolyl)] -benzene
ppm	parts per million
PPO	2,5-diphenyloxazole
psi	pounds per square inch
Δ^5-	Δ^5-pregnenolone; 5-pregnen-3α-ol-20-one
RIA	radioimmunoassay
rpm	revolutions per minute
s.c.	subcutaneous
SKF-525A	2'-diethylaminoethyl-2,2-diphenylpentanoate
T	testosterone; 4-androsten-17β-ol-3-one
technical DDD	90% p,p'-DDD + 10% o,p'-DDD

LIST OF ABBREVIATIONS AND COMMON NAMES (Cont.)

technical DDT	80% p,p'-DDT + 15-20% o,p'-DDT
TLC	thin layer chromatography
TP	testosterone-17-proprionate
TVBE	tail vein-bleeding under ether anesthesia

CHAPTER 1

INTRODUCTION

This investigation was undertaken to determine the feasibility of using the measurement of specific endocrine functions as a general screen for the effects of foreign compounds on reproduction and viability. Its primary purpose was to generate approaches which might be utilized to shorten current toxicological screening procedures by augmenting or supplanting the multigenerational tests now used to measure effects on reproduction. Secondarily, its goal was to establish whether 1,1,1-trichloro-2-(2-chlorophenyl)-2-(4-chlorophenyl)-ethane (o,p'-DDT) has any effect on the steroidogenic endocrine tissues of the developing male rat and if so, what mechanism was involved in producing the effect.

The experiments to be described do shed some light on the secondary questionsand as a result imply the utility of at least a portion of the approach for use in screening protocols. Of equal importance, perhaps, they indicate the potential for utilization of xenobiotics, such as o,p'-DDT, to produce pathologic states which can provide information about the normal development and function of the endocrine system that is not easily obtained by known procedures. Finally, these experiments indicate both a direction for future investigations on the mode of action of o,p'-DDT in the steroidogenic endocrines and a crude estimation of how much data of this type must be amassed to generate conclusions of use in the legal regulation of synthetic chemicals.

In order to understand the experiments detailed in Chapters 3-5 and their relation to the objectives an exposition of some of the background literature and the resultant experimental approaches and reasoning is necessary. To that end I will briefly discuss the present models of steroidogenic endocrine function and development and the known metabolism and biological effects of DDT.

I. Steroidogenic Endocrine Function:

The physiologic "purpose" of the steroidogenic endocrines appears to be the production of the steroid hormones; the major synthetic paths are indicated in Figure 1. The active forms of these hormones typically show effects on target tissues possessing intracellular steroid receptor proteins (1,2). Usually these effects are trophic in nature and lead to growth, proliferation and/or differentiation of the target tissue. However, in the case of the hypothalamic centers and at least in some of the pituitary cell types (3) the steroids act by suppressing release of the releasing hormones (-RH, -RF) or the trophic hormones. In this latter manner they indirectly control their own production. Likewise, by effecting the functions of the adrenals and/or kidneys and liver (4,5), they may ostensibly exert some control over their own inactivation as well.

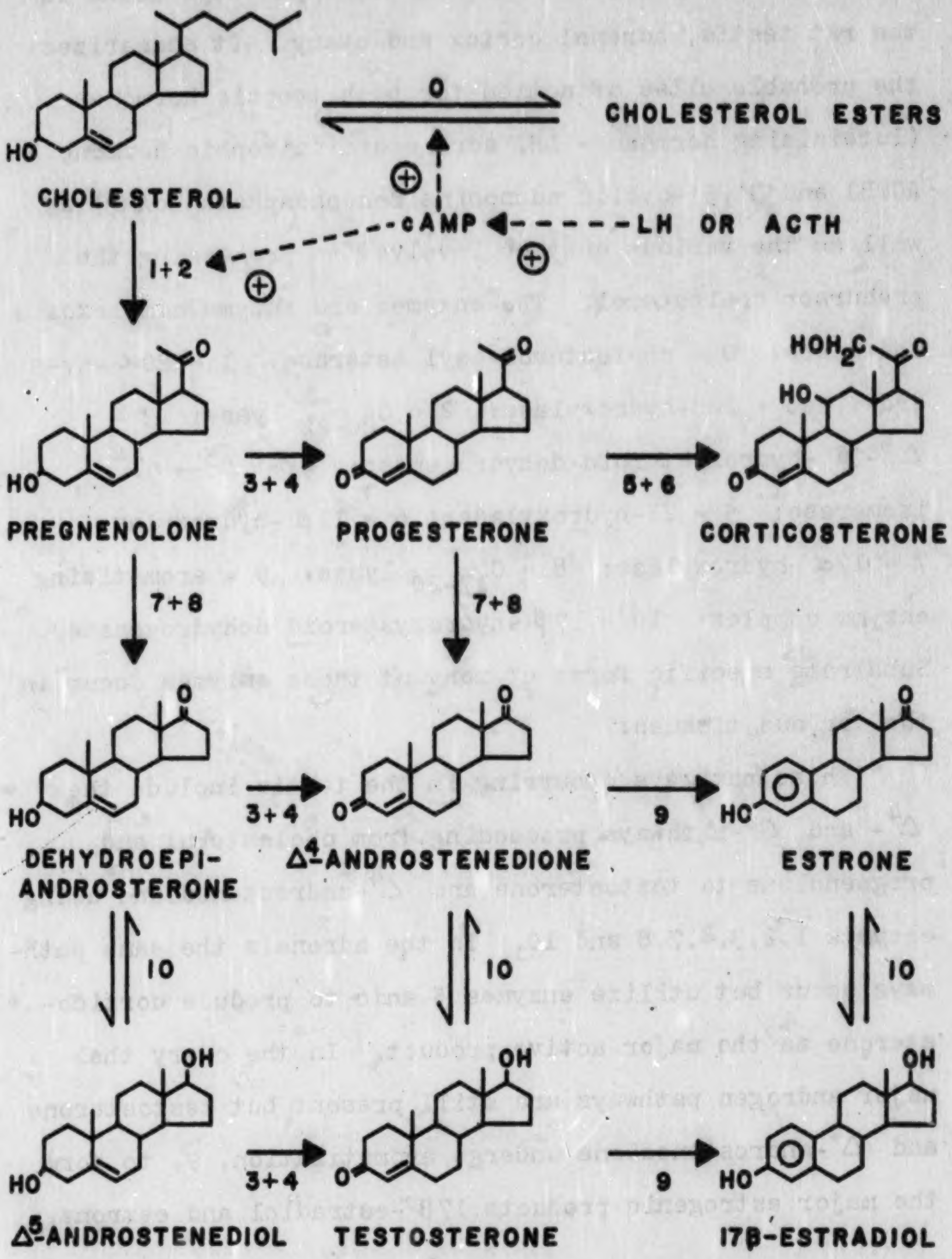
Figure 1. Major Steroid Pathways in the Rat

Shown is a composite of the major pathways found in the rat testis, adrenal cortex and ovary. It summarizes the probable sites of action for both trophic hormones (luteinizing hormone - LH, adrenocorticotrophic hormone - ACTH) and 3',5'-cyclic adenosine monophosphate (cAMP) as well as the various enzymes involved in processing the precursor cholesterol. The enzymes and enzyme complexes shown are: 0 = cholesterol-acyl esterase; 1 = 20α-hydroxylase + 22R-hydroxylase; 2 = C_{20-22} lyase; 3 = Δ^5-3β-hydroxysteroid dehydrogenase; 4 = $\Delta^5 \rightarrow \Delta^4$ isomerase; 5 = 21-hydroxylase; 6 = 11β-hydroxylase; 7 = 17α-hydroxylase; 8 = C_{17-20} lyase; 9 = aromatizing enzyme complex; 10 = 17β-hydroxysteroid dehydrogenase. Substrate specific forms of many of these enzymes occur in the various tissues.

Those pathways occurring in the testis include the Δ^4- and Δ^5-pathways proceeding from cholesterol and pregnenolone to testosterone and Δ^4-androstenedione using enzymes 1,2,3,4,7,8 and 10. In the adrenals the same pathways occur but utilize enzymes 5 and 6 to produce corticosterone as the major active product. In the ovary the major androgen pathways are still present but testosterone and Δ^4-androstenedione undergo aromatization, 9, to form the major estrogenic products 17β-estradiol and estrone.

FIGURE I

MAJOR STEROID PATHWAYS IN THE RAT


HO
CHOLESTEROL
0
CHOLESTEROL ESTERS
⊕
cAMP
LH OR ACTH
⊕
1+2
⊕
O
HO
PREGNENOLONE
3+4
O
O
PROGESTERONE
HOH2C
O
HO
5+6
O
CORTICOSTERONE
7+8
7+8
O
HO
DEHYDROEPI-
ANDROSTERONE
3+4
O
O
Δ4-ANDROSTENEDIONE
9
O
HO
ESTRONE
10
10
10
OH
HO
Δ5-ANDROSTENEDIOL
3+4
OH
O
TESTOSTERONE
9
OH
HO
17β-ESTRADIOL

Subcellularly, steroids are formed in the adrenals and gonads from cholesterol or cholesterol esters by the action of enzymes localized in the lipid droplets (cholesterol-acyl esterase), mitochondria (C_{20-22} lyase, 18- and 11β-hydroxylases), endoplasmic reticulum (3β- and 17β-hydroxysteroid dehydrogenases, $\Delta^5 \rightarrow \Delta^4$-isomerase, C_{17-20} lyase, 17α- and 21-hydroxylases and aromatizing enzymes) and cytosol (20α-hydroxysteroid dehydrogenase) (6). These multienzyme pathways and the indicated compartmentalization allow subcellular modulation of steroid synthesis. They also impose numerous potential sites for the action of modulators, e.g., specific enzyme regulation, membrane modification, blockage or stimulation of cosubstrate (reduced nicotinamide adenine dinucleotide phosphate - NADPH) production, etc. This latter point becomes particularly important and complex when the precise mode of action of an extracellular agent is in question.

The rate of synthesis of the steroids appears to be largely dependent on circulating trophic hormone levels and the state of activation of intracellular adenylate cyclase which is proximally associated with the trophic hormone receptors. Stimulation of the cyclase, or inhibition of 3',5'-cyclic nucleotide phosphodiesterase (7), ultimately leads to protein synthesis generally and subsequently to steroidogenic enzyme synthesis. In addition, direct activation of cholesterol-acyl esterase and the cholesterol side-chain cleavage complex occurs acutely upon stimulation of the cyclase (8). Thus, the rate of steroidogenesis is both acutely and chronically controlled by enzyme levels and activation states which are in turn determined by trophic hormone levels.

Serum trophic hormone levels are controlled by the rate of synthesis and release of hypophysial trophic hormone stores. In their turn, the stores of these protein hormones depend on the activation of cellular protein synthesis and on cAMP concentrations which are controlled by the level of certain peptides, the trophic hormone releasing hormones (factors), in the hypophysial portal blood (3,9). Further modulation occurs by the direct feedback actions of the steroids as mentioned above. Once more, as in the case of the trophic hormones acting on the adrenals or gonads, an extracellular signal, -RH, controls the synthesis and release of a hormone.

Finally, the release of the releasing hormones is controlled by neurons which contain steroid receptors and which are associated with the neurons which produce and release the releasing hormones into the portal vessels. At this point, however, the modulation by steroid hormones is inverse, and the negative feedback loop of classic endocrinology is completed (Figure 2) (3,9,10).

Throughout this complex feedback system the ultimate effects on steroid target tissues are subject to modulation by parameters such as receptor levels, Ca^{++} levels, blood flow, multiple hormone synergisms, etc., which are in turn

Figure 2. Schematic Diagram of the Steroidogenic
Endocrine System of the Male Rat

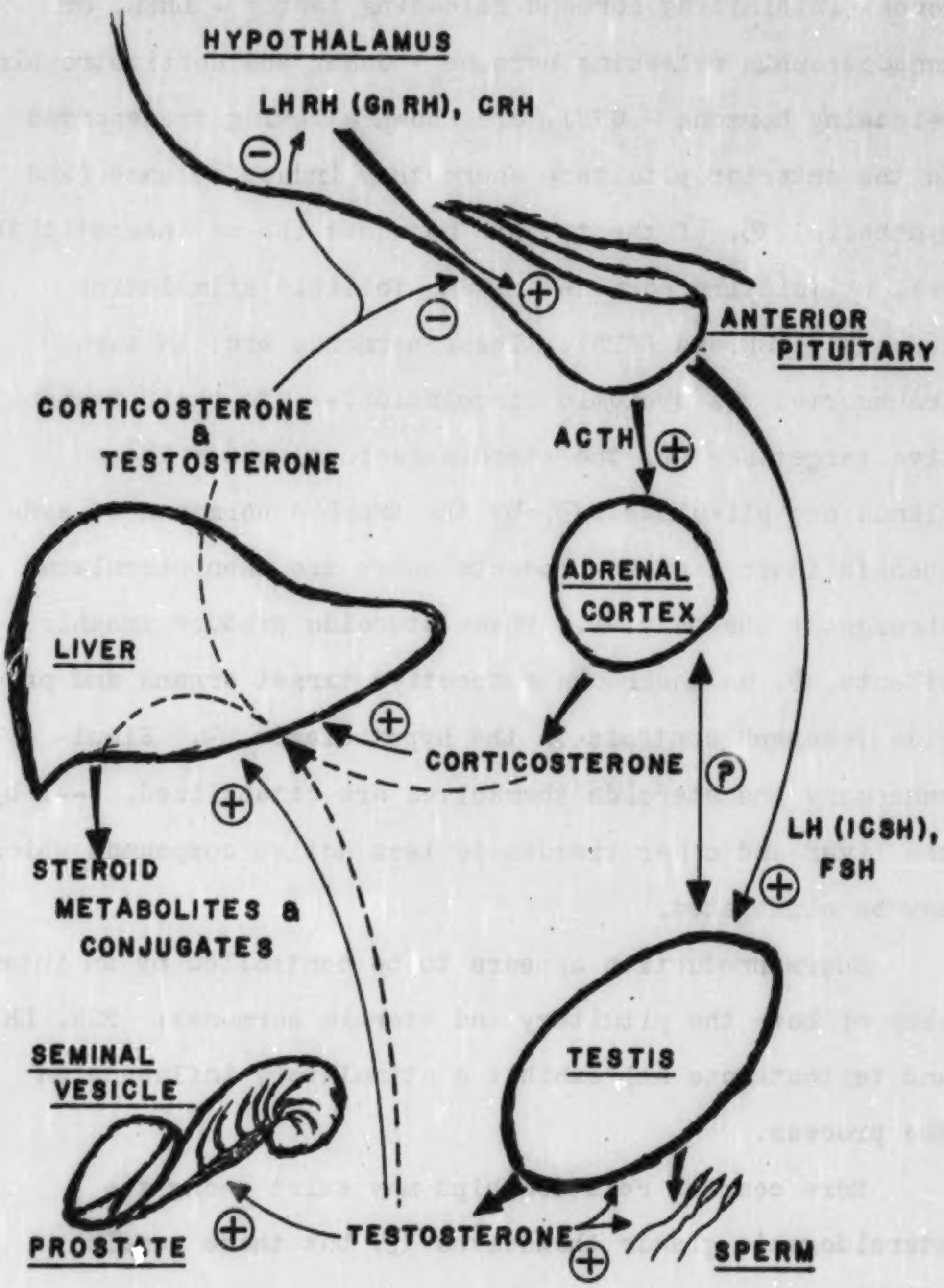
The products of the hypothalamus, the releasing hormones (luteinizing hormone releasing factor - LHRH, or gonadotrophin releasing hormone - GnRH, and corticotrophin releasing hormone - CRH), are shown as being transported to the anterior pituitary where they induce release (and synthesis), ⊕, of the trophic hormones (LH or interstitial cell stimulating hormone - ICSH, follicle stimulating hormone - FSH, and ACTH). These hormones are, in turn, transported via systemic circulation, →, to their respective target organs, the steroidogenic glands. These glands are stimulated, ⊕, by the trophic hormones to synthesize their steroid products which are then circulated throughout the body, →. These steroids produce trophic effects, ⊕, on their own respective target organs and provide feedback controls on the hypothalamus, ⊖. Simultaneously the steroids themselves are catabolized, --→, by the liver and other tissues to less active compounds which may be eliminated.

Sperm production appears to be controlled by an interplay of both the pituitary and steroid hormones; FSH, LH and testosterone all exhibit a stimulatory influence on the process.

More complex relationships may exist among the steroidogenic glands themselves, ?⃝, but these remain to be clarified.

FIGURE 2

SCHEMATIC DIAGRAM OF THE STEROIDOGENIC ENDOCRINE SYSTEM OF THE MALE RAT


HYPOTHALAMUS
LHRH (GnRH), CRH
⊖
⊕
⊖
ANTERIOR PITUITARY
CORTICOSTERONE & TESTOSTERONE
ACTH ⊕
ADRENAL CORTEX
LIVER
⊕
CORTICOSTERONE
?
⊕
LH (ICSH), FSH
⊕
STEROID METABOLITES & CONJUGATES
SEMINAL VESICLE
TESTIS
PROSTATE ⊕
TESTOSTERONE
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determined by age, sex, nutrition, health, other physiological states and/or the external environment. The feedback loops, which help maintain organismic homeostasis, and the sensitivity of these loops to large numbers of diverse modifying influences appear to be quite suitable as indicators of the unknown effects of exogenous agents on an organism. Since the initial phenomenological question is always whether an agent does or does not exhibit a measurable effect, the fact that exact etiology of an elicited alteration in a hormone feedback loop is unclear is immaterial. If the elicited change is potentially capable of altering the viability and/or fertility of the animal involved the regulatory screening question has been answered. The complexity of endocrine control systems does present problems in defining exact modes of xenobiotic action but it also provides experimentally exploitable elements not found in systems contained within single cells or organs.

In addition to allowing experimental manipulation of these feedback loops the possibilities of exploiting temporal changes is also available. Studies of the time course for the maturation of these loops, in both sexes, and of the cyclical alteration of the loops caused by pregnancy, menstruation or estrus, provide opportunities to explore stages of varying sensitivity to environmental influence. They thus allow identification of the stage(s) in life during which the exogenous agent being tested produces the most profound and/or lasting effects.

Previous work in the general area of toxicity on the steroidogenic endocrines/has concentrated on the adult rat and its responses to long term feeding or acute injection of a variety of drugs, pesticides and other synthetic chemicals. Conney _et al_. concentrated on the effects of barbiturates and pesticides on the clearance of steroids from systemic circulation (11-18) and on the capacity of target organs (uterus and prostate) to bind steroids in the presence of the synthetic chemicals (19-23). Their results have indicated that steroid metabolism and function is indeed sensitive to agents which induce hepatic microsomal metabolism; the steroids are metabolized more quickly and more extensively than normal in treated animals. Further, this same group along with several others (24-27), have shown that some chemicals, e.g., o.p'-DDT and polychlorinated biphenyls (PCB's) are capable of specifically blocking the uptake of steroids by their target organs even in the absence of hepatic induction. Rybakova _et al_. (28-30) have shown that the pesticides DDT and Sevin are capable of increasing pituitary levels of gonadotrophins in male rats fed contaminated food for long periods of time. Ottoboni has demonstrated lengthened reproductive life span in female rats fed DDT (31) while Ware and Good and Harr _et al_. have shown a decrease in fertility and fecundity in mice and rats treated with Mirex and dieldrin (32,33). Acute blockage of the ovulation surges of both LH and estrogen in the rat have been shown with barbiturates (34) given on the morning of proestrus. Ethyl ether acutely raises the serum levels of FSH and LH in rats (35,36). An extensive literature exists on the inhibitory action of DDT and its analogs (especially 2,2-dichloro-1-(2-chlorophenyl)-1-(4-chlorophenyl)-ethane (o,p'-DDD)) on the adrenal cortices of man (37-41), the dog (42-47) and guinea pig (48,49) and on the lack of this effect on the adult rat (50,51). ACTH release in the rat was increased by dieldrin in a chronic manner unaccounted for by stress (52). Finally, Heinrichs and Gellert have demonstrated cystic ovarian development, persistent vaginal estrus and decreased serum LH levels in response to adult castration in female rats injected neonatally with o,p'-DDT (53,54).

These last investigations (53,54) have a unique and important character. They demonstrate a permanent alteration of a steroidogenic endocrine control system and, together with the "imprinting" experiments discussed below, imply the acute sensitivity of these control systems early in life, either perinatally in the rat and mouse or in utero in most other species. Of equal importance, this sensitive point occurs coincidentally with complete maternal dependence and with the time of most effective transfer of diffusable and lipid soluble chemicals from mother to offspring (55,56). Since the functioning of this system after perinatal exposure may be one of the most sensitive indicators of the potential toxicity affecting the reproduction and the viability of species and individuals, the coincidence of these two conditions, impressionability and dependence, indicate the need to examine endocrine function subsequent to such exposure.

II. Steroidogenic Endocrine Development: Imprinting

Because the immature steroidogenic endocrine system appears in the above case (53,54) to be so vulnerable to the presence of an exogenous agent, demonstrating permanent changes in endocrine status or response, the need to review steroidogenic development becomes clear. Since most of the studies have been done on the rat I shall concentrate there.

Some of the most classical studies concerning the development of the sex steroid systems relate to the development of the brain, its sex specificity and control properties. These studies, reported by Harris (57) utilized the technique of ectopic implantation. Ovarian grafts were made to the anterior chamber of the eye and vaginal grafts to subcutaneous sites. By surgically and chemically manipulating the steroid status of the host animal, e.g., neonatal or adult castration plus steroid injection, etc., it was possible to determine the basic outline of hypothalamic sex differentiation. As a result of these studies and those reported by Barraclough (58-60) and Gorski (61) it became established that there are two hypothalamic centers which control gonadotrophin release and which, in turn, are affected by the presence of circulating sex steroids.

First, there is a tonic center, anatomically located in the arcuate-ventromedial nuclear area, which in adulthood controls the tonic release of GnRH, and, subsequently, LH and FSH. This center is subject to negative feedback inhibition by circulating testosterone (in males) or 17β-estradiol (in females). Additionally, it remains largely intact even if doses of exogenous estradiol or testosterone are given during the perinatal period, 1-10 days of age.

The second center, the cyclic center, is associated with the preoptic area of the hypothalamus and controls the gonadotrophin surges seen near the time of ovulation in the mature female. It differs from the tonic center both in that it apparently responds positively to circulating estrogen levels above an unknown threshold level by increasing release of GnRH, LH and FSH and in that it is effectively eliminated by either perinatal administration of exogenous steroids or by the testosterone endogenously produced by the intact neonatal male. It is this elimination of the cyclic center by exposure to androgens or estrogen during the critical perinatal period which has led to the concept of "imprinting". A multitude of manipulations of the castrate and intact state with steroids, neuronal lesions and surgery now support the imprinting concept (61-63). A summary of some of them is presented for comparative purposes in Table 1.

Since perinatal steroid status manipulations, such as those above, alter all those steps controlled by the hypothalamus as well as the hypothalamus itself imprinting has obvious pleotropic effects. Most prominent of these effects are phenotypic changes in external genitalia, growth and sexual behavior (57-61), all of which are associated with varying degrees of sterility. But imprinting is not necessarily a clear-cut phenomena; the degrees of abberation are quite dependent on the steroid dosages administered and the timing. For example, 100% of a group of female rats injected with 1250 ug of testosterone propionate on day 5 postpartum showed sterility as adults while 70% showed sterility with a dose of 10 ug (59). Or, as another example, castration of male rats beyond 5 days of age blocks normal estrus cycles in implanted ovaries while similar males bearing implants after castration prior to day 5 do demonstrate normal estrus cycles. The implications are that endocrine manipulations may alter both the cyclic and tonic centers (63), the differences in end result being a matter of the comparative degree of alteration of the two sites.

More subtle changes which may involve sites other than the two centers defined thus far also take place as has been shown by Gustafsson et al. in their studies of specific steroid metabolic enzymes in the liver (64-68).

Table 1

Description of Neonatal "Imprinting"[1] of the Hypothalamus[2] of the Rat

Gonadal Status	Age	Sex (at birth)	Functional Cyclic Center?	Functional Tonic Center?	Normal Development?
I. Uninjected Pups					
Intact	0-15 Days	Male	+[3]	+	+
		Female	+	+	+
Intact	5-15 Days	Male	−	+	+
		Female	+	+	+
Intact	Adult	Male	−	+	+
		Female	+	+	+
Neonatally Castrated	Adult	Male	+	+	−
		Female	+	+	−
II. Neonatally Injected with Estrogen or High Levels of Testosterone					
Intact	Adult	Male	−	+ ?	−[4]
		Female	−	±	−
Neonatally Castrated	Adult	Male	−	±	−
		Female	−	±	−

Table 1 (Cont.)

III. Neonatally Injected with o,p'-DDT					
Intact	Adult	Male	-	±	+ ?
		Female	±	±	-
Neonatally Castrated	Adult	Male	?	+ ?	-
		Female	?	?	-

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[1] "Imprinting" is defined as fixation of a biochemical or physiological state or developmental direction; depending on the parameter measured, it may be observed at any time between 5 and 15-30 days of age.

[2] The tonic and cyclic centers of the hypothalamus are those neurons or nuclei which control tonic or cyclic release of gonadotrophins.

[3] Symbols used indicate established normality, = +, or abnormality, = -, and uncertain functionality = ±. Unknown functionality or conclusions based only on the results in this thesis are indicated as ?.

[4] The degree of abnormality is dependent on dosage and time of administration after birth.

Perinatal treatment of females or neonatally castrated males with high doses of testosterone, 5α-dihydrotestosterone, 5α-androstanediol or estradiol all yielded hepatic alterations tending toward the intact male phenotype. Similar treatments of the castrate adult male, however, offer a more complex picture, some activities being altered toward the female or castrate phenotypes, others being unaffected (64,68). The data are consistent with enzymatic activities being classified into groups in which control is: (1) not exerted by the presence of steroids, (2) actively exerted by the presence of androgens, and/or (3) imprinted by the presence of neonatal steroids.

Beyond these controls by hypothalamic mechanisms - which may include alterations in neuronal growth and association (60) - imprinting by steroids may also occur by direct effects on peripheral tissues. Diminished specific uptake of labelled estradiol into pituitary and uterus of adult rats neonatally injected with androgens or estrogens was shown by Flerkó et al. (69) and McGuire and Lisk (70). Similar decreases in testosterone uptake into the pituitary were found by Simmons (71) in adult male rats neonatally injected with estradiol and castrated two days prior to testing. These experiments seem reminiscent of the findings in the genetically determined androgen-insensitive rat described by Bullock and Bardin (72). This rat lacks the steroid receptor proteins necessary for effective specific uptake of androgens into target tissues and consequently exists as a pseudohermaphrodite. Since changes like these, or others involving receptors for trophic hormones (73), may exhibit temporal and dosage dependencies somewhat different from those of hypothalamic imprinting per se, they may well be the biochemical mechanisms ultimately responsible for the broad spectrum of morphological and physiological changes seen in adult animals neonatally exposed to androgenizing or estrogenizing influences at differing times and dosages.

III. Steroidogenic Endocrine Development: The Normal Time-Course

Morphologically the testis demonstrates two generations of testosterone-producing cells (the interstitial or Leydig cells). These cells, occupying the interstitium between the tubular (sperm-producing) elements of the testis, exist at birth and are histochemically demonstrable until roughly 5-7 days of age. They largely disappear at this time and either regenerate or become reactivated at approximately 3 weeks of age. Production of potent spermatozoa in the tubules begins to occur at roughly 45 days of age; sexual maturity as measured by sexual potency, peripheral organ enzyme activities and serum hormone levels is reached by roughly 50-60 days of age (74,75).

Steroid production in the first generation of Leydig cells appears quite similar to that occurring in the adult and is sensitive to exogenously administered human chorionic gonadotrophin (hCG), i.e., LH (76). The testis which exists between the first and third week of life appears steroidogenically inactive and insensitive to exogenous LH because testosterone levels fall even in the continuous presence of serum LH (77). This may, in fact, be due to the diminished number of Leydig cells present (76) although other schemes involving the coincident absence of the glucocorticoids (see below) have been advanced in the face of the constitutivity of the testicular LH receptors (78,79). Full adult steroidogenic capacity is gradually reached during the period following weaning (at roughly three weeks of age) and prior to maturity (80,81), the rise proceeding most rapidly from about 40 to 60 days of age. Accessory gland development closely correlates with the increasing serum levels of active androgens produced during this period of maturation as do the levels of certain enzyme activities found in peripheral tissues such as the liver (4,5).

Serum hormone levels during the developmental period have been measured by several groups (77,82,83). Levels of LH appear highly variable prior to approximately three weeks of age after which they stabilize slightly below adult levels until the onset of maturity at which the slight rise to adult levels occurs. FSH demonstrates a stable plateau at roughly adult levels until about three weeks of age. At this time the levels rise about two-fold and plateau until 40-50 days of age after which they fall back to adult levels. On the hypothalamic level, GnRH concentrations begin to slowly rise immediately after birth and finally reach the adult plateau at about 50-60 days of age (84). Throughout development the feedback loop remains sensitive to exogenous LHRH (82) or castration (85-87) implying that it is functional and is only modified or modulated differently during the adult period, the change taking place during the transitional state, puberty.

Development in the female exhibits a distinctly different pattern. Within about 10 days after birth the ovaries demonstrate aggregation of several follicular cells with each egg cell and progression of the initial meiotic division of the egg cells themselves to the dictyate stage of prophase I. The oöcytes appear to randomly begin enlargement, apparently initiating the events which would eventually lead to ovulation in the adult ovary. Prior to the cyclical events which occur during the estrus cycle, however, those oöcytes which began development and stimulated their associated follicular cells to divide and form small follicles undergo degeneration long before reaching the ovulatory state. This atresia seems to be associated with unfavorable concentrations of estradiol, LH and/or FSH (88,89) and may reflect immaturity of the thecal portion of the ovary.

Slow maturation of the thecal, i.e., interstitial or stromal, elements of the ovary ensues over the 5-6 weeks after birth. These steroidogenic elements show sensitivity to endogenous LH and FSH producing serum estradiol peaks subsequent to serum LH peaks (77) and supporting uterine weight gain (90). Near 35-40 days of age the theca reaches full maturity and is capable of playing its role in the cyclic events of normal estrus. Its production of estradiol, perhaps as a result of the presence of adult titers of gonadotrophins, becomes sufficient to cause full differentiation of the female accessory tissues. This results in the opening of the vagina, the maturation of the uterus and the initiation of female sexual behavior.

Steroid synthesis during the immature phase is localized in the thecal tissue, estrogen being the major product. After the first ovulation, however, the post-ovulatory follicle forms a temporary gland and site of progesterone production, the corpus luteum; similar temporary glands subsequently generate the cyclic changes in serum progesterone seen during the female cycle.

Trophic hormone production by the female hypophysis differs from that of the male. FSH is markedly elevated at birth and remains so until roughly three weeks of age when it drops to a plateau of 1/3 to 1/4 the early level. It continues there until the periodic rises associated with ovulation begin to occur at about 5-6 weeks of age (77). LH, on the other hand, shows nearly the same picture as in the male; highly variable levels exist until three weeks of age followed by a plateau near adult diestral (basal) levels until the onset of cyclicity near 40 days of age (77). Hypothalamic GnRH rises slowly from birth until 4-6 weeks postpartum when the plateau of maturity is reached (84). The feedback controls are all operative as can be seen by the abrupt response of serum LH to perinatal ovariectomy or steroid injection (85,87) or by the halt in ovarian and uterine growth caused by administration of anti-gonadotrophin anti-sera (90).

In the female, puberty and final maturity seem closely associated with ovarian development and/or ovarian mediated changes in the neural-hypophysial control centers (91). Conversely, in the male, changes in testicular sensitivity to LH caused by high FSH levels appear to play the dominant role (92,93). Though they begin similarly, the control systems for reaching maturity appear quite different for the two sexes.

Adrenal cortical development in the rat follows a similar time course in both sexes for roughly the first three weeks postpartum. The glomerulosal layer is well defined at the exterior of the cortex and appears stable throughout the developmental timecourse. The cortical tissue within the glomerulosa appears compact and undifferentiated at birth with the reticular and fasicular zones being morphologically indistinguishable (94). No definitive perimedullary fetal or x-zone is present, as it is in the mouse, but steroidogenesis does occur as has been shown by Milković and Milković (95). The response to stress appears blunted for several days following birth but at no time is it entirely absent (96). Further, this response is sensitive to hypothalamic control by CRH and to hypophysial control by ACTH from the 18th fetal day onward as has been shown in experiments using natural and experimental anencephally and hypophysectomy (97). In spite of this functional intactness the adrenal cortex exhibits a regression in volume, cell size and steroid content immediately after birth and does not regain mature functionality until sometime between 2-3 weeks of age (96). Beyond this point development in the sexes begins to differ because estrogen produces a positive trophic action on the cortex resulting in larger adrenals with higher corticosterone output in the female. This difference has been implicated in the onset of female puberty through a direct influence of the adrenal on the ovary (98) although the exact mechanism is at yet unclear. During the period of 3-5 weeks postpartum the fasicular and reticular zones become differentiated, formation of vascular elements and tissue cords becoming visible histologically. Finally, by 5-6 weeks of age the adult diurnal fluctuations of corticosterone output become established (98) and maturation of this system is also completed.

IV. Metabolism and Physiological Effects of DDT:

The rat was chosen as the animal model for this work because of the wealth of information available on its normal steroidogenic functioning and development and because of its potential susceptibility to perturbation of those normal conditions. Choice of the model compound was based on a review of the known properties of widely used synthetic chemicals. DDT stands out as one of the few chemicals which has been extensively examined from essentially all facets: chemical, toxicological, pharmacological and ecological. And yet, the extent of its biological effects, to say little of its mode of action, remain incompletely defined, as do many of the factors which cause it to demonstrate specific toxicities, e.g., reproductive toxicity.

In speaking of DDT it must first be borne in mind that the commercial chemical, technical DDT, is actually a mixture. Most analyses are in general agreement with those of Haller et al. (99), finding 70-80% 1,1,1-trichloro-2,2-bis-(4-chlorophenyl) ethane (p,p'-DDT), 15-20% o,p'-DDT plus small amounts of 2,2-dichloro-1,1-bis(4-chlorophenyl) ethane (p,p'-DDD), o,p'-DDD and other synthetic-reaction byproducts. Because of this predominance of p,p'-DDT in the mixture a large proportion of the literature on the insecticide has been generated using pure preparations of p,p'-DDT. Still, it must be said that all the major components have been tested for, and found to have, biological effects.

Metabolically, in mammals, p,p'-DDT has been found to undergo either dehydrohalogenation of the side-chain to form 2,2-dichloro-1,1-bis-(4-chlorophenyl) ethylene (p,p'-DDE) or dechlorination of the side-chain to form p,p'-DDD and subsequently side-chain oxidation to form 2,2-bis-(4-chlorophenyl) acetic acid (p,p'-DDA) which may be conjugated (100,101). It has also been shown to be susceptible to nonenzymatic reductive dechlorination to p,p'-DDD catalyzed by agents such as lake water, reduced porphyrins, water-logged soil and boiled pigeon liver (102-104). Yet even though conversion to p,p'-DDD and then to p,p'-DDA seems to be a ubiquitous possibility the conversions occur slowly enough for both DDT and its metabolites to accumulate, sometimes drastically, in biological tissues. This accumulation, which is responsible for the large majority of the effects seen in non-target species, is due not only to DDT's slow metabolic conversion to excretable products, but also to its high lipid solubility, a property which it shares with its two major neutral metabolites DDD and DDE.

The proportions and levels of the DDT derived residues found in mammalian tissues is highly dependent on factors determined by the species, age, sex, nutritional status and drug-history of the animal involved (105-112). These factors not only determine the distribution of body fat, the major storage tissue for the residues, but also, more importantly, the activity of the hepatic microsomal enzymes. These enzymes, which have been demonstrated to be involved in the active metabolism of p,p'-DDT (100,113, 114), are similar to, if not the same as, those involved in the breakdown of drugs such as phenobarbital or steroids such as testosterone (12-15, 115-122). Furthermore, in most mammalian and avian species studied the enzymes are inducible by drugs (barbiturates) or by DDT itself (11, 13-15,19-21,115-122). Such induction leads to decreases in the total pesticide load (123,124) and in the DDE/DDD ratio but it also increases metabolism of normal body constituents such as steroids and vitamin D (125-129). It is this latter consideration which is particularly worrisome in non-target species since it can result in serious hormonal and nutritional imbalances.

Another major factor influencing the form and levels of the stored insecticide residues is the presence of gut flora. McCully et al. established the differences in DDE/DDD ratios in rats, sheep, chickens, rabbits and guinea pigs given DDT by several different routes (130). Their conclusions agree well with other investigations which have found that DDT metabolism involves extensive enterohepatic circulation and is highly sensitive to the presence of gut microflora (105,131). These microbes function under largely anaerobic conditions and, like those in anaerobic soils, catalyze the formation of DDD from DDT

(132,133). This catalysis overcomes what appears to be a rate limiting step in DDT breakdown, allowing more rapid catabolism to DDA and shunting DDT away from the formation of the inert storage residue, DDE.

Finally, levels of residue retention are, of course, dependent on the extent of exposure. In the case of low, 1 -50 ppm, exposures in the food of rats, the quantity of total DDT residues climbs to a plateau over the course of 17-23 weeks and decreases over the course of about 25-30 weeks after exposure has been discontinued (134). This pattern of residue levels is paralleled, as expected, by microsomal drug metabolizing activity and is sensitive to agents which further induce or surpress hepatic microsomal activity (106,107,129) or which mobilize stores of body fat (108).

Most of the previous statements concerning metabolism are also applicable to the other chemicals found in technical DDT. The normal metabolite p,p'-DDD is broken down to p,p'-DDA, as mentioned above, and yields no residues of p,p'-DDE (100,132,133,135,136). On the other hand, o,p'-DDT, as is shown in Figure 3, is not only subject to side-chain decomposition but is also degraded by way of ring hydroxylation reactions (51,137,138). These latter reactions are predictable on the basis of the open para-position in the ortho-substituted ring. This extra metabolic "handle" and the higher polarity of the resultant hydroxylated metabolites can largely explain the higher

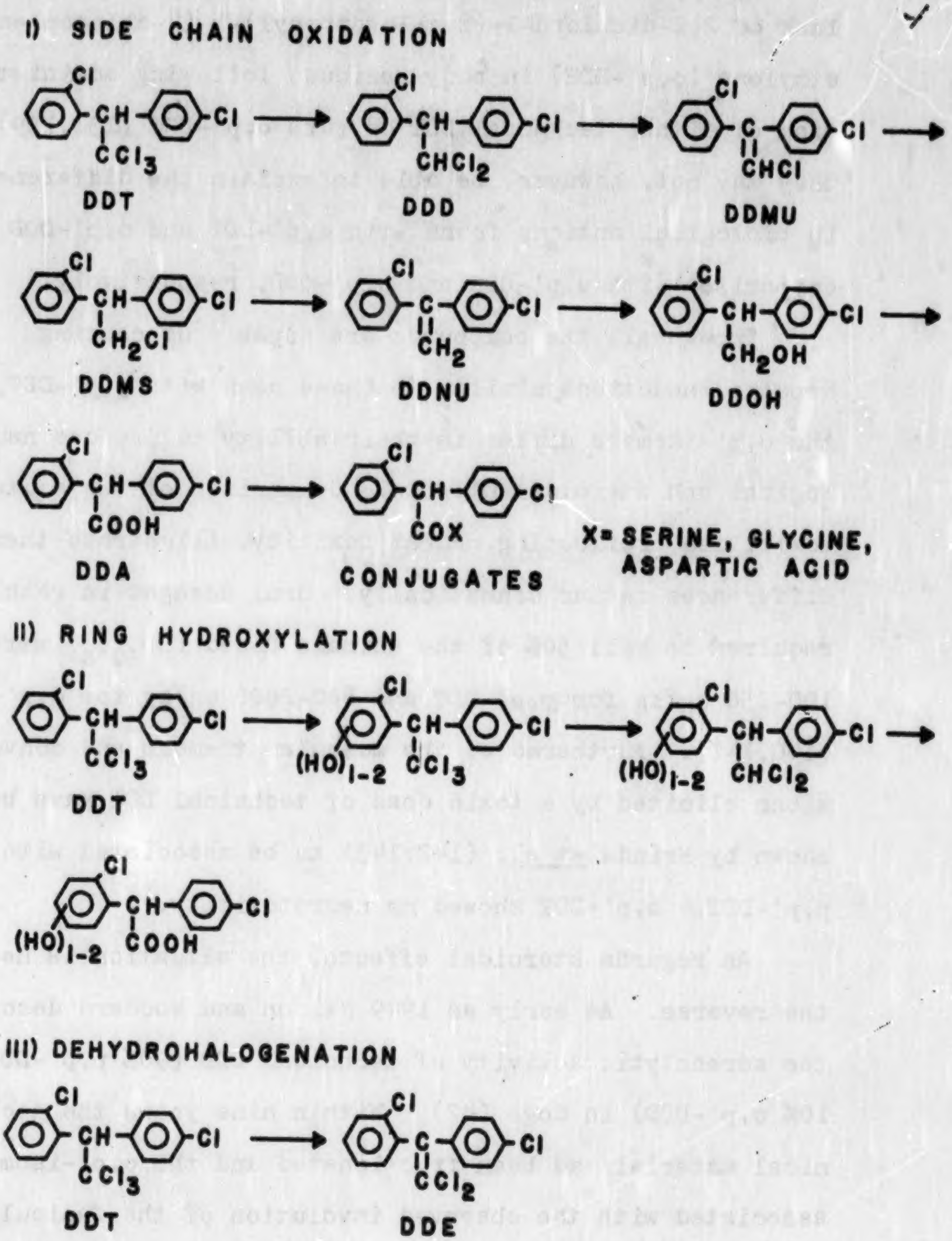
Figure 3. Major Metabolic Routes of o,p'-DDT

The known routes of DDT metabolism are depicted as separate and parallel pathways. In mammalian systems pathways I) and II) cross in such ways as to yield a variety of hydroxylated intermediates with the side-chain in various stages of oxidation or conjugation. Pathway III) appears to be independent for the p,p'-isomer of DDT producing a major storage form of the pesticide; however, for the o,p'-isomer hydroxylation and other routes seem to be favored since o,p'-DDE residues are detectible only in minor quantities.

The specific enzymes involved in the pathways are largely unknown. The P-450 complex does play some role since it is induced by DDT and stimulates the elimination of DDT residues after induction by other agents.

FIGURE 3

MAJOR METABOLIC ROUTES OF o,p'-DDT


I) SIDE CHAIN OXIDATION
Cl
CH
Cl
CCl3
DDT
CHCl2
DDD
C
CHCl
DDMU
CH2Cl
DDMS
CH2
DDNU
CH2OH
DDOH
COOH
DDA
COX
CONJUGATES
X= SERINE, GLYCINE, ASPARTIC ACID
II) RING HYDROXYLATION
(HO)1-2
III) DEHYDROHALOGENATION
CCl2
DDE

metabolic rate of o,p'-DDT and o,p'-DDD in comparison with the p,p'-isomers. Furthermore, they can also explain the lack of 2,2-dichloro-1-(2-chlorophenyl)-1-(4-chlorophenyl) ethylene (o,p'-DDE) in body residues following administration of either technical DDT or pure o,p'-DDT (131,139). They may not, however, be able to explain the differences in biological actions found with o,p'-DDT and o,p'-DDD in comparison with p,p'-DDT and p,p'-DDD, respectively.

Though all the compounds are capable of causing hepatic inductions similar to those seen with p,p'-DDT, the o,p'-isomers differ in their ability to produce neurological and steroidal effects. Comparison of the acute toxicities, reflecting neural toxicity, illustrate these differences rather dramatically. Oral dosages in rats required to kill 50% of the animals dosed ($LD_{50's}$) were 100-250 mg/kg for p,p'-DDT and 800-2000 mg/kg for o,p'-DDT (140,141). Furthermore, the muscular tremors and convulsions elicited by a toxic dose of technical DDT have been shown by Hrinda et al. (142,143) to be associated with p,p'-DDT; o,p'-DDT showed no neurotoxicity.

As regards steroidal effects, the situation is nearly the reverse. As early as 1949 Nelson and Woodard described the adrenolytic activity of technical DDD (90% p,p'-DDD, 10% o,p'-DDD) in dogs (42). Within nine years the technical material had been fractionated and the o,p'-isomer associated with the observed involution of the fasicular and reticular cortical zones and with the associated decrease in urinary and cirulating 17-hydroxy- and 17-keto-steroids (45). After similar activity was shown in several species including man and the purified material became available, o,p'-DDD began to be used for the treatment of adrenal hyperfunction as it occurs in Cushing's syndrome and adrenal carcinoma.

The other outstanding example of steroidal effect is the innate estrogenicity of o,p'-DDT. In a study of DDT effects on White Leghorn cockerels published in 1950 Burlington and Lindeman (144) demonstrated what appeared to be an estrogen-like effect of prolonged exposure to technical DDT. They found a marked decrease in testicular growth and in development of secondary sex characteristics and attributed the effects to a configuration of p,p'-DDT which superficially resembles the synthetic estrogen diethylstilbestrol. A conflicting report based on work with female rats appeared in 1952 (145), but it was not until 1968 that Bitman et al. (146) and Welch et al. (22) conclusively demonstrated that only o,p'-DDT was intrinsically estrogenic. These later studies showed that not only did o,p'-DDT have a trophic effect on the oviducts of injected chickens and quail (146) but it also was capable of increasing wet weight, glycogen content and ^{14}C-glucose uptake into lipid, proteins and RNA in immature or ovariectomized adult rat uteri (22, 147). Subsequent studies by Singhal et al. (148) showed that the uterotropic response in ovariectomized rats also involved enhancement of gluconeogenic and hexosemonophosphate shunt enzymes; the enzymatic enhancement was inhibited by actinomycin D or cycloheximide. This report also indicated that acute administration of o,p'-DDT combined with 17β-estradiol produces a somewhat additive effect. Notwithstanding this last result, other studies (26,27) have shown that o,p'-DDT is capable of effectively competing for uterine cytosolic estradiol receptors in vitro at levels of 1-10 ppm; such levels are within the reported tissue levels of DDT under normal environmental exposure (149). Still later experiments by Gellert et al. (53,54,150,151) have demonstrated that o,p'-DDT can decrease serum LH in ovariectomized adult female rats and is even capable of causing appearance of persistent-vaginal-estrus in adult rats injected with 3 mg neonatally. This last effect, perhaps the most striking, has been demonstrated to be dose-dependent and specific to o,p'-DDT (if all of its aspects are included). It represents a starting point for the studies described in this thesis.

It must also be noted that a large body of literature, coming mostly from the laboratories of A. H. Conney, has outlined the complicating factor in any in vivo studies concerning the steroidal actions of DDT and its analogs, i.e., the induction of hepatic metabolism (11,14-21,23,39, 118,120,122,127-129,143). This series of papers has demonstrated that chronic doses of pesticides or drugs are both capable of inducing the hepatic enzymes responsible for catabolizing circulating steroids of all classes. The work of Peakall (152), albeit in ring doves, directly demonstrates reductions in circulating estradiol levels upon chronic feeding with p,p'-DDT. That such is also the case in the rat and mouse has been indirectly shown by the fact that uterine ^3H-17β -estradiol uptake and the uterotropic response to estradiol are both blocked by chronic exposure to halogenated hydrocarbon insecticides (18). Similar results have also been shown for androgen uptake and response in rat seminal vesicles (23) or mouse prostate (25). That microsomal induction may occur throughout the postnatal life of the rat has also been demonstrated (109,153). All the above observations taken together require that in vivo experiments on the mode of action of pesticides, especially in regard to their actions on steroidogenesis, be interpreted with the possibility in mind that indirect effects mediated by hepatic steroid metabolism are responsible for the effects seen.

The mode of toxic action of DDT and its analogs in mammals may well be related to its demonstrated actions in insects. Holan (154) briefly described a molecular model of the membrane site to which DDT binds in insect nerve. This model was based on molecular-dimension calculations and on an extensive series of structure-function relationships including at least 57 analogs of p,p'-DDT. The final picture of the binding of DDT involved insertion of the trichloroethyl-tail into a pore in the lipid portion of the membrane with the aromatic rings extending into the protein layer and forming π-bonds to the protein molecules. The pore to which DDT was bound is associated with Na^+ transport; treatment with the insecticide results in atypical and continuous nerve impulses indicative of perturbed Na^+ efflux. Other work has indicated coincident or subsequent changes in K^+ and/or Ca^{++} transport in both insect and mammalian species and in both nerve and muscle preparations (155). Obviously, if the compound effects such universal elements of membrane function as membrane potential and cation transport it is not surprising that Hrinda et al. (143) have demonstrated increased levels of cAMP in tissues taken from rats treated with DDT or its analogs. This is especially so in view of the postulations of cation involvement in the production and actions of cAMP put forward by Rasmussen (9).

The foregoing discussion has emphasized how much we know and implied how much we do not know about the DDT complex (technical DDT), one of the best characterized of the synthetic chemical mixtures. In spite of the great humanitarian services DDT performed after its rediscovery in 1939, a great public outcry arose concerning DDT's safety after the publication of Rachel Carson's Silent Spring in 1962 (156). This outcry, combined with the professional questions which stemmed from the papers listed in the above discussions and from ecological studies such as Hickey's Peregrine Falcon Populations, Their Biology and Decline (157), resulted in the banning of DDT for use in the United States in 1972. Still, its environmental persistence (158), global mobility (159,160) and extensive use outside of the U.S. continue to make it a material of some ecological importance. Because o,p'-DDT was implicitly included in the ban and the furor which brought it about and because it has known effects which include precisely the type of alterations which could best be probed by the approaches I have proposed for screening other materials, I chose to use o,p'-DDT in these investigations.

Having chosen the endocrine system of the rat and o,p'-DDT as objects of the experimentation, more specific questions arose. First, the phenomenological question of the presence of a measurable effect begged a second question which had mechanistic ramifications; what part of the endocrine system or its control loop is affected? This question forced an experimental design capable of answering both the question of the existence of an effect and the question of the focus of action of the chemical under study. Therefore, the object of the experimentation had to be more fully defined. Being that earlier reports have generally indicated little reproductive effect of DDT on the adult rat (161,162) and that several studies had been published on the effects of neonatal injection with o,p'-DDT in the female rat (53,54,150,151), the choice was made to examine the results of neonatal exposure on the function of the endocrine system of the male rat. The somewhat simpler adult hormone pattern of the male as compared to the female also made the male system more attractive than the female. But the question, "On what?" required even further definitions. It must be understood that the question of looking for an effect on the organism as a whole or on a specific subsystem within the organism required a broad investigation employing a variety of measurements. Furthermore, the measurements had to probe both structure and function in order to ascertain if subtle but still consequential changes had taken place. In regard to the endocrine system, this emphasis on scope required that a variety of measurements be made on both the gonadal-hypothalamo-hypophysial and the adrenal-hypothalamo-hypophysial systems. That is, observations including morphology, histology and hormone secretions had to be coupled with function tests, akin to glucose-tolerance tests, such as responses to castration or exogenous LHRH. Obviously, though the investigation was undertaken to determine the existence of an effect it may also give insight into the later question of mechanism of action. The question of an effect "On what?" was therefore answered in these studies by noting any differences in the results of the measurements used in treated and untreated animals.

It is evident from the previous discussions that the perinatal period is, at least in the rodent, a period of either ambiguity or preparation in the steroidogenic control systems. It is therefore a period of great sensitivity to outside influences in both the male and female. Because of this, and because the neonatal detoxification systems are not as active as they are in the adult (5), the potential for exogenous agents such as o,p'-DDT to influence reproduction and/or viability seems greatest if exposure occurs perinatally as was proposed in these experiments. It seemed reasonable that if a model of interference with the steroidogenic control loops was to be used in screening for the effects of foreign compounds, direct introduction of the materials into test groups could generate the desired answers. If, however, the specific effects of natural exposure was to be used or questioned, introduction must occur via the mother, since neonates are normally completely dependent on her. In these investigations both routes of exposure were examined.

In the case of either exposure, measurements were made in both intact and neonatally castrated rats to ascertain if some of the changes found in the female (53,54,150, 151) could be found in the proportedly neurologically similar neonatally castrated male (57-61). Furthermore, it was hoped that such parallel experiments might elucidate the role of the testes in any changes observed in the intact rat. The measurements performed included body and organ weights, organ histologies, serum hormone levels under normal and stressed conditions and pituitary hormone levels. Studies on in vitro testicular steroid transformations were also initiated and spawned the generalized techniques and computer program detailed in Chapter 6 and Appendix I. A general schematic of the approach and treatments used is shown in Figure 4.

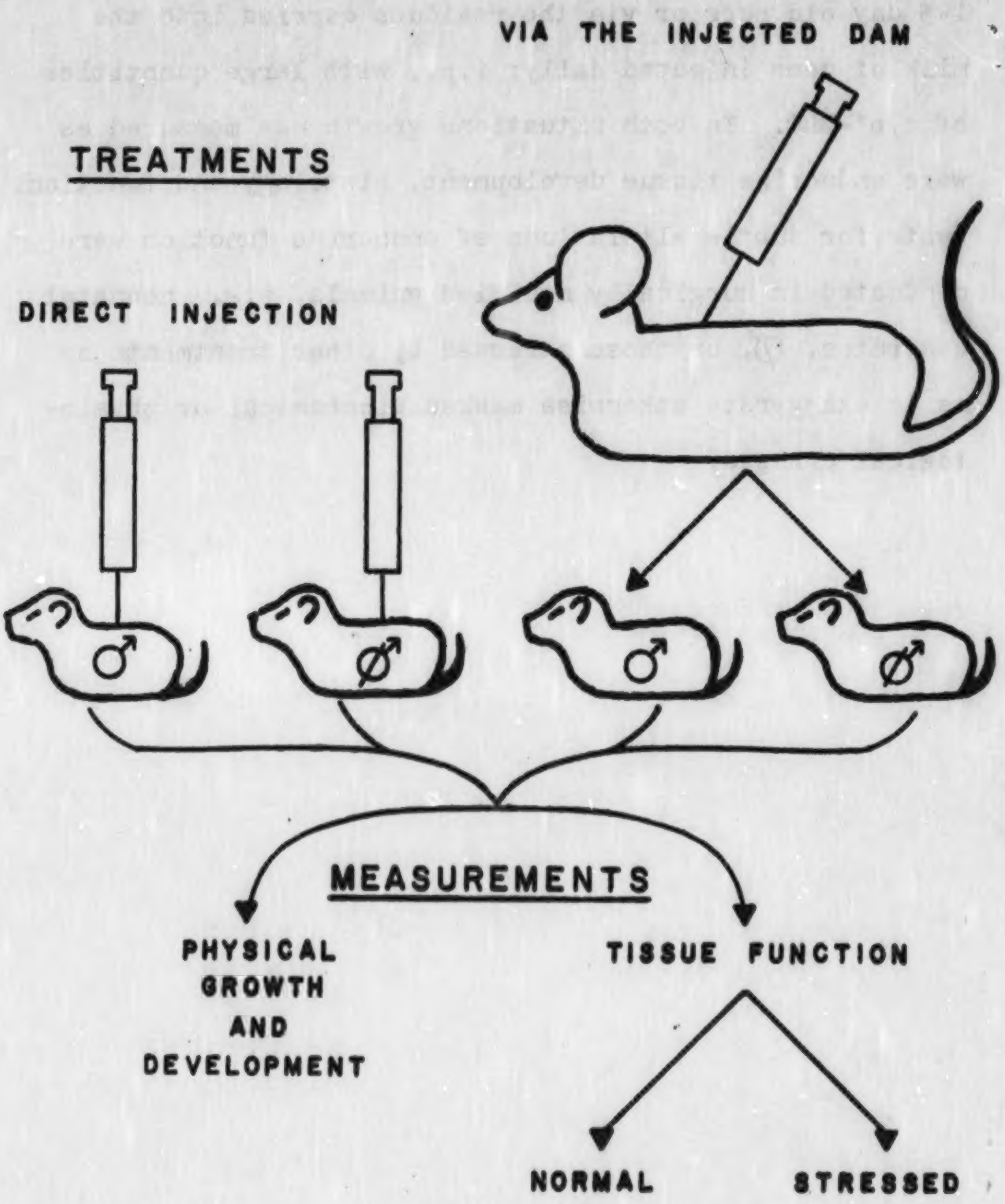
The observations described in this thesis are meant to answer the experimental question posed: "What is the effect, if any, of neonatal exposure to o,p'-DDT on the steroidogenic endocrine systems of the male rat?" Some of the later studies may also shed some light on the locus of action of the chemical because they were designed to fix the actual location of the effects of o,p'-DDT more firmly than the initial studies; they attempted to answer the question "Exactly where does it act?" Even the question of when the changes occur was partially addressed by studies which examined development in conjunction with function. It is with these questions in mind that the experiments will be described and related to the primary objective, i.e., design of a general procedure for screening foreign compounds for effects on reproduction and viability.

Figure 4. Schematic of Approach and Treatments

Treatment was either via direct s.c. injection into 1-5 day old pups or via the residues carried into the milk of dams injected daily, i.p., with large quantities of o,p'-DDT. In both situations growth was measured as were endocrine tissue development, histology and function. Tests for subtle alterations of endocrine function were conducted in surgically modified animals, e.g., neonatal castrates, Ø, or those stressed by other treatments so as to exaggerate otherwise masked biochemical or physiological changes.

FIGURE 4

SCHEMATIC OF APPROACH AND TREATMENTS


VIA THE INJECTED DAM
TREATMENTS
DIRECT INJECTION
♂
Ø♂
♂
Ø♂
MEASUREMENTS
PHYSICAL GROWTH AND DEVELOPMENT
TISSUE FUNCTION
NORMAL
STRESSED

CHAPTER 2

MATERIALS AND METHODS

I. Animals:

All rats used in these experiments were Sprague-Dawley purchased from Spartan Laboratories, Hazlett, Michigan. They were housed in 30 cm x 30 cm x 15 cm nesting boxes on wood shavings from birth until weaning and thereafter in 35 cm x 60 cm x 15 cm cages with wire mesh floors. Caging was in groups of 6-8 dependent on age. All animals were allowed free access to water and food (Purina Rat Chow). A light-dark cycle of 12/12 (6.00 - 18.00 EST) was maintained. Room temperature and humidity varied somewhat with the season, but normal values were approximately 25°C and 60%. Cages were cleaned every 2-3 days. Medication was only used when serious outbreaks of respiratory infection occurred in or near the experimental groups or when animals were exposed to heavy or repeated ether anesthesia. In any cases in which medication was used, all test and control groups were treated equally with Terramycin dissolved in the drinking water (50 mg/ml).

When neonates were to be used, the pregnant females were placed in nesting boxes roughly one week before delivery. After delivery litter sizes were adjusted to meet the needs of the experiment, usually 6 or 8 pups per dam. Litters were maintained, by addition of pups if necessary, as near to the original number as possible until weaning. Pups were allowed access to the dams until 25 days of age by which time natural weaning was complete in all litters (163).

Neonatal castrations were performed under cold anesthesia according to the procedure of Pfeiffer (164). Following the surgery and warming to adult body temperature, the pups were returned to their dams. Adult castrations were performed under ether anesthesia. A short midventral-scrotal incision was made and followed by two small lateral incisions through the tunica vaginalis of each testis. The testes were expressed through the incisions and the spermatic artery, vein and the vas deferens were tied off with sterilized cotton thread. The testes were severed below the constriction and the wound closed with wound clips. Adult castrates were placed on Terramycin for at least 3-5 days after the surgery.

Blood samples were obtained from animals either after decapitation, in which case trunk blood was collected, or under light ether anesthesia by tail-vein bleeding (TVBE). The latter procedure was chosen over heart-puncture, catheterization or tail-vein bleeding without anesthesia for the following reasons. First, I found heart puncture, during which many animals were killed by faulty punctures, to be a less predictable process. Since many of these animals required 2-3 months to obtain I was conservative in their use. Second, catheterization is normally only acceptable for bleedings carried out for less than 3-5 days and requires a moderate amount of surgery to insert the cather. Because a large number of animals were to be bled during periods which exceeded one week catheterization was discarded. Finally, after several attempts at bleeding by tail-vein without anesthesia the ability of the rat to constrict blood flow to the tail became obvious. Because blood samples of 1.0 ml or more were needed for the measurements to be made anesthesia was necessary.

TVBE was carried out by placing the rat on an ether-moistened cotton pad in a metal can equipped with a glass cover. After 45-60 seconds the rat was sufficiently anesthetized to be placed in a restraint cage, to have a small (about 5 mm) fragment of the tail cut off with a razor blade, and to have 1-3 ml of blood collected in a test tube prior to regaining full consciousness. Including the tying off of the tail with a loop of cotton cord, the time from introduction into the ether chamber to placing the animal into a holding cage was 3-5 minutes.

Tissue samples were collected after the animals were weighed, stunned, decapitated and bled. If they were to be examined histologically they were weighed, divided and placed into 10% neutral-buffered formalin (165).

Animal weights were determined on a triple beam balance to within ± 0.5 g. The adrenals and pituitaries of all of the animals and the testes and accessory glands of young or steroid-treated rats were rinsed in a modified

Ringer's solution (Buffer I, Table 3), blotted and weighed on a Sartorious semi-automatic milligram balance accurate to ± 0.03 mg. Kidneys and livers from all the animals and the testes and accessory glands from older rats were rinsed and blotted as above and weighed on a Mettler top-loading balance accurate to ± 0.01 g.

II. Chemicals:

All chemicals utilized were obtained from commercial sources. Miscellaneous solvents, salts, acids and bulk chemicals were obtained from Mallinckrodt, Inc., Fisher Scientific Co., Merck Chemical, J. T. Baker Chemical Co. and Matheson, Coleman and Bell Manufacturing Chemists. All water used in solutions or washes, etc., was double-distilled.

DDT analogs were purchased as 99^{+}% pure from Aldrich Chemical Co., Inc. Subsequent analysis by thin layer chromatography (TLC) demonstrated single spots for all of these chemicals; however, gas chromatography (GC) later showed impurities in the o,p'-DDT, and p,p'-DDD. Bitman et al. had analyzed Aldrich o,p'-DDT in 1971 as being 98.7% pure, with 1.1% p,p'-DDT contamination (166). Analysis of the o,p'-DDT used in these experiments showed 98.5% o,p'-DDT and 1.5% p,p'-DDT even after recrystallization from several solvent systems (acetone/water, ethanol, hexane). Contamination of p,p'-DDD was with minor (< 1%) amounts of o,p'-DDD.

Labelled steroids, ^3H- and ^{14}C-, were purchased from Amersham/Searle Corp. Cold steroids were obtained from Sigma Chemical Co. and Schwartz-Mann. Purity was verified for all labelled steroids by TLC followed by radioscanning at high sensitivity on a Vanguard Model 880 strip scanner. Three solvent systems were used: benzene, 8:2-benzene: acetone, and chloroform. Labelled steroids were used at the original specific activity determined by the manufacturer: 18.6 Ci/mmole, $[7\ n\text{-}^3H]$-Δ^5-pregnenolone; 52 mCi/mmole, 4-^{14}C-dehydroepiandrosterone; 59.4 mCi/mmole, 4-^{14}C-testosterone; 60 mCi/mmole, 4-^{14}C-Δ^4-androstenedione; 53 mCi/mmole, 4-^{14}C-Δ^5-pregnenolone; 61.0 mCi/mmole, 4-^{14}C-progesterone; 61 mCi/mmole 4-^{14}C-17α-hydroxyprogesterone. They were diluted to appropriate volumes, i.e., the correct number of counts per volume, with 1:1-benzene:ethanol or ethanol and stored under refrigeration. Unlabelled steroids were similarly checked for purity by TLC and subsequently recrystallized from ethanol and/or acetone; purity was then checked by TLC, GC and melting point. Cold steroids were stored at room temperature in brown glass, and their solutions in ethanol or benzene-ethanol were refrigerated. Corticosterone solutions were made up in redistilled ethanol and water and stored for no more than one week under refrigeration. Cholesterol-3-propyl ether (CPE) was purchased in highly purified form (verified by GC) from Sigma and was stored at -20°C. CPE solutions were made up in 1:1

benzene:ethanol and stored under refrigeration. Testosterone-17-propionate and 17β-estradiol-17-valerinate were also purchased from Sigma and were used as supplied.

Aldrin was obtained from K & K Laboratories, Inc. and purified by recrystallization from ethanol and hexane after washing with methanol and acid. Purity was verified by appearance of a single peak upon GC.

Sodium phenobarbital and 2'-diethylaminoethyl-2,2-diphenylpentanoate (SKF-525A) were donated by the laboratories of Dr. M. J. Coon and Dr. H. H. Cornish, respectively. The chemicals were dissolved in physiological saline by addition of acid or base and titrated to pH 7-8 prior to use in injections.

Pyridine nucleotides ($NADP^+$ and NADH), glucose-6-phosphate (G-6-P), glucose-6-phosphate dehydrogenase from Baker's or Torula yeast (G-6-P DH) and bovine serum albumin (BSA) were purchased from Sigma. Synthetic LHRH was obtained from Calbiochem. All these biologicals were diluted in modified Ringer's solution or physiological saline immediately prior to use. Storage of the crystalline materials was at -20°C.

Refined sesame oil and dimethyl sulfoxide (DMSO) were obtained from Fisher Scientific and used as obtained. Glass-distilled hexane purchased from Burdick and Jackson Laboratories, Inc. was also used as received. To remove fluorescent impurities, dichloromethane from Mallinckrodt or Fisher Scientific was allowed to stand over concentrated sulfuric acid for several days, then washed with sulfuric acid, bicarbonate and water. The wet solvent was dried over anhydrous sodium sulfate and distilled through a Vigreaux column (the 39°C fraction was collected). Similarly, ethanol was distilled after refluxing with 2,4-dinitrophenylhydrazine and hydrochloric acid to remove fluorescing impurities.

Precoated silica gel G TLC plates were purchased from Brinkman Instruments, Inc. and thoroughly washed with methanol prior to use. Gas Chrom Q and silicone GC coatings were purchased from Applied Science Laboratories. GC column packings were prepared using filtration and fluidizer techniques (167).

Scintillation chemicals, 2,5-diphenyloxazole (PPO) and 1,4-bis-[2-(5-phenyloxazolyl)]-benzene (POPOP), were obtained from Research Products International, Corp. The scintillation cocktail used was 6 g PPO plus 0.25 g POPOP per liter of toluene and was based on the data of Bush and Hansen (168) for nonquenched systems.

The ovine-LH (oLH), radioiodinated oLH and anti-rabbit gamma globulin utilized in the radioimmunoassay (RIA) procedures were obtained from the Reproductive Endocrinology Program of the Department of Pathology, The University of Michigan. The anti-oLH used was a dilution of the Niswender anti-serum (169) and was also obtained from the Pathology Department. These biologicals were used as supplied.

III. Treatments:

All direct chemical treatments were by injection. Steroids and o,p'-DDT were dissolved in sesame oil for subcutaneous (s.c.) injection into neonatal animals. Such injections were done on the day of birth and repeated on the following four days; all these injections were volumes of 0.05 ml. The concentrations used are given in the experiments. Injections of o.p'-DDT into nursing dams, to indirectly administer the chemical to the pups, were given intraperitoneally (i.p.). These injections were begun on the day of parturition and were continued daily until the day of weaning (day 25). All injections were either 0.1 ml of 50 mg of o,p'-DDT dissolved in DMSO or 0.1 ml DMSO alone. The solutions of DDT in DMSO were nearly saturated and were warmed prior to injections.

The quantities of steroids given to neonates were based on the results of imprinting experiments previously conducted (59) and were chosen to be near the lowest effective single doses. These doses were divided into five equal parts, one part of which was given in 0.05 ml of sesame oil on each of days 1-5 of age; daily doses were 40 ug 17β-estradiol-17-valerinate and 200 ug testosterone-17-propionate. The amounts of DDT injected into neonates was meant to cover a range from nearly environmental exposure levels to levels near those found effective by Gellert et al. (54) in imprinting the neonatal female.

The daily dose of o,p'-DDT given the dams was estimated to be approximately 1/10th of an LD_{50} dosage.

In later experiments involving the response of serum-LH to adult castration, a test of the negative feedback loop was performed by injecting testosterone several hours prior to bleeding. The dose of testosterone administered, s.c., in 0.1 ml of sesame oil was determined, on the basis of the work of Hutchison and Goldman (170), to be capable of partially suppressing castrate levels of serum LH without driving them down to normal intact levels.

The response to LHRH was measured by injecting a large (171), 1 ug, dose of synthetic LHRH into intact rats. The injection was in 0.1 ml of physiological saline and was performed 20-60 minutes prior to bleeding. This period has been shown to coincide with maximal serum-LH concentrations by previous workers (9,172-174).

The hepatic microsomal inducer, phenobarbital, and the inhibitor of microsomal induction, SKF-525A, were administered to neonatal rats i.p. in 0.05 ml of physiological saline. The doses used were based on the work of Levin et al. (17) and Harbison (175). Phenobarbital was given daily in two injections of 100 ug each, spaced 12 hours apart, for the first five days of life. SKF-525A was given as a single daily injection of 125 ug over the same five day period.

The amount of DDT actually absorbed by rat pups suckling injected dams was determined during these studies and is discussed in Chapter 4.

IV. Measurements:

A. Organ Weights:

Body and organ weights were measured as above and the percentage of body weight attributable to each organ was calculated for each animal. Means, standard deviations and standard errors were then computed for both absolute weights and percent of body weight for each treatment group and each organ. Mean values were then compared statistically by applying unpaired Student's t-tests. Significance throughout this investigation was considered to be attained only if the chance probability of the observation in question was less than 5%.

B. Organ Histologies:

Tissue fragments fixed in 10% neutral buffered formalin were prepared for observation by the Histology Laboratory of the Department of Pathology, The University of Michigan. Common histological preparation was followed, i.e., alcohol/xylene dehydration, paraffin embedding, sectioning, staining, xylene/alcohol clearing and mounting (165). The tissues were sectioned at 7-10 mu and stained with hematoxylin-eosin. Observations were made at 5-100 power using a Zeiss photomicroscope with a polarizing light source and Nikon objectives. Subjective visual observations were recorded for each tissue and slide.

When differences between treatment groups became apparent the slides were reexamined to verify the differences. The adrenals, which showed the most marked changes due to o,p'-DDT administration, were photographed using the same microscope and Eastman Kodak Co. color photomicrography film (ASA 16/13 DIN). The resulting slides were processed into prints by Kodak. Between-group comparisons of the frequencies of a given histologic observation were made by using the X^2 test for a 2 x 2 table.

C. Serum Corticosterone Measurements:

Serum corticosterone was measured by a modification of the procedures of Mattingly (176) and Silber *et al*. (177). Figure 5 shows a schematic diagram of the protocol utilized.

Upon decapitating an animal a trunk-blood sample was collected into a 10 or 20 ml beaker. This was allowed to clot on ice before being transferred to a tube and centrifuged for 20 minutes at approximately 5000 rpm (Variac setting 50) in a Servall tabletop centrifuge. The serum was decanted and stored at -20°C. Just prior to extraction the serum was thawed and 1.0 ml was mixed with 2.0 ml of purified ethanol (all solvents were purified - see II. Chemicals). The protein precipitate was removed by centrifugation under the same conditions as previously. To a measured volume of 0.5-1.0 ml of supernatant in a screw-capped test tube, 1.0 ml of doubly-distilled water and 5.0 ml of glass-distilled hexane were added. This

Figure 5. Protocol for Measuring Serum Corticosterone Levels, Modified from Mattingly and Silber

Tests of both the Mattingly (176) and Silber (177) procedures for serum corticosterone led to the outlined protocol. The alkali wash used by Silber was found unnecessary if the hexane wash was retained and the reagents were all purified prior to use in the assay.

Figure 5

Protocol for Measuring Serum Corticosterone Levels, Modified from Mattingly and Silber

Fresh Blood Sample

Centrifuged 20 min at 5000 rpm in a tabletop centrifuge; serum decanted

↓

Serum

Frozen

↓

1.0 ml Thawed Serum

2.0 ml EtOH added, mixed then centrifuged as above; aqueous EtOH decanted

↓

0.5-1.0 ml Aqueous EtOH

1.0 ml H_2O and 5.0 ml hexane added, mixed, then centrifuged as above; hexane aspirated off

↓

Washed Aqueous EtOH

5.0 ml CH_2Cl_2 added, mixed and centrifuged 10 min as above; aqueous EtOH aspirated off; CH_2Cl_2 dried with anhydrous Na_2SO_4 or $MgSO_4$

↓

Dry CH_2Cl_2 Extract

Stored for 1-2 days under refrigeration before reaction or reacted immediately by adding 3.0 ml of 7:3-H_2SO_4:EtOH, mixing for 20 sec, centrifuging for 30 sec and aspirating off the CH_2Cl_2 extract

↓

Fluorescent Mixture

13 min after addition of the H_2SO_4:EtOH emission read at 535 nm using 475 nm excitation and a C-70 filter (cutoff = 510 nm)

solution was mixed for at least 20 minutes on a test tube rotator or a metabolic shaker before being centrifuged as before. The upper, hexane, layer was carefully aspirated off and the lower, alcoholic, layer reextracted with 5.0 ml of dichloromethane. Again mixing was for at least 20 minutes on a tube rotator or a metabolic shaker; centrifugation was for 10 minutes at 5000 rpm. The upper, alcoholic, layer was carefully aspirated off and the lower, dichloromethane, layer was dried with anhydrous Na_2SO_4 or $MgSO_4$. After drying, the tubes could be tightly covered with Parafilm or teflon and stored under refrigeration for up to 48 hours prior to assay without altering the results. Though this short-term storage was possible assays were completed as soon as possible after extraction.

The actual measurement was as follows. The dry dichloromethane extract was added to 3.0 ml of a 7:3 mixture of concentrated sulfuric acid:ethanol in a glass stoppered conical centrifuge tube. This was Vortexed for 20 seconds and centrifuged for 30 seconds on a Clay Adams tabletop centrifuge operated at a speed setting of 1-2. The upper, dichloromethane, layer was aspirated off. At 13 minutes ± 30 seconds the fluorescence at 535 nm of the alcoholic sulfuric acid solution was measured in a 1.0 cm quartz fluorescence cuvette using 475 nm excitation light and a 510 nm cutoff, exit filter (Corning C-70). The instrument used was an Aminco-Bowman fluorimeter. The measured fluorescence was recorded and later corrected for the background fluorescence of blanks run simultaneously. Calculation of concentration was based on the background-corrected fluorescence of standards run simultaneously with the unknowns.

Initial assays demonstrated that the use of an alkali wash of the dichloromethane extract was unnecessary since similar results were obtained in assays done on the same sera without the wash. The correctness of the use of corticosterone standards diluted in water was verified by showing that a strictly additive relationship occurred in samples measured with and without the addition of a standard sample of corticosterone. It was also shown by the parallelism of a standard curve with a set of serial dilutions of a serum pool, Figure 6. A water blank was used in all assays; the stock standard solution was 2 ug of corticosterone per milliliter in water.

The mean index of precision of the assay, λ, was ±13.45%; mean sensitivity was 0.969 ± 0.027 log units of fluorescence per log unit of concentration, in ug/ml; the mean limit of detection, defined as blank plus λ, was 0.0062 ug/ml. A measurement of recovery done in pentuplicate on extracted and unextracted standards indicated total recovery to be 74 ± 2%. It should be noted that the parameters given are means and that the values have improved during the course of experimental repetition so that the value of λ, particularly, may be better reflected by the value of ±9.07% obtained in the last assay.

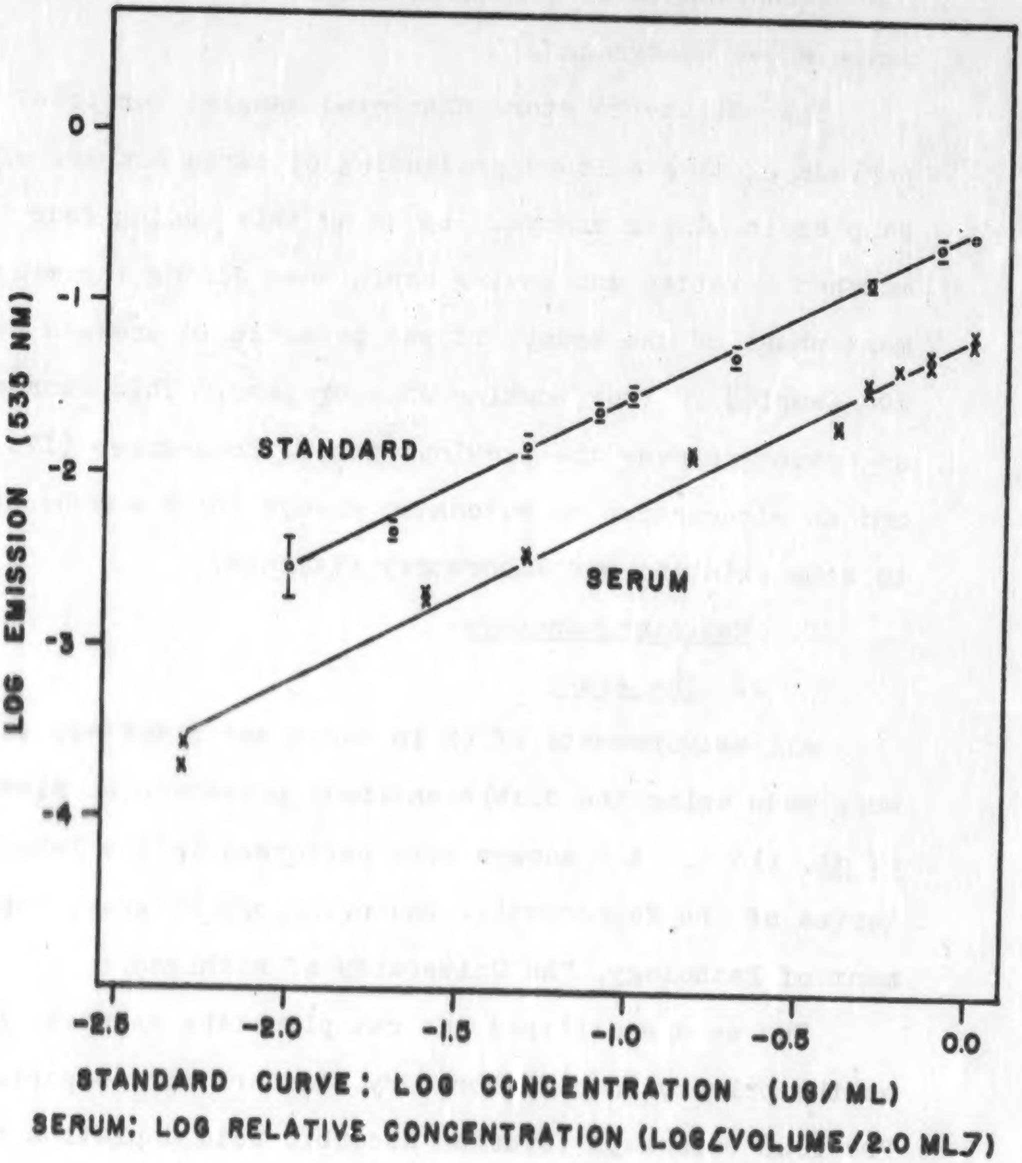
Figure 6. Verification of the Sulfuric Acid Fluorescence Assay for Serum Corticosterone: Parallelism of the Standard with Serially Diluted Rat Serum

Log emission at 535 nm versus log concentration for the standard or log relative concentration for serially diluted serum yield straight lines with slopes of 0.995 ± 0.022 and 1.034 ± 0.029, respectively. The standard curve is plotted as mean log emission ± 1 standard deviation (where errors are covered by the circle they are not shown); the serum curve shows the individual measurements in a duplicate determination. The serial dilutions were made starting with a 2.0 ml volume of serum.

Since the standard contained only corticosterone diluted in water the parallelism with the serially diluted serum implies that the assay is specific over this concentration range (178). Any contaminant would have to demonstrate 2 different partition coefficients equalling those of corticosterone and have a fluorescence intensity at 535 nm similar to that of the standard.

FIGURE 6

VERIFICATION OF THE SULFURIC ACID FLUORESCENCE ASSAY FOR SERUM CORTICOSTERONE: PARALLELISM OF THE STANDARD WITH SERIALLY DILUTED RAT SERUM



The specificity of the assay has been previously shown by Silber et al. (177) and is supported by the parallelism (178) of the standards and serially diluted unknown shown in Figure 6. Interference of DDT metabolites in treated animals was also eliminated as a source of error when measurements of fortified blanks showed no fluorescence above background.

The ability to store extracted samples for brief periods of time allowed processing of large numbers of samples in single assays. By doing this, using four matched cuvettes and having assistance during the measurement phase of the assay, it was possible to process over 200 samples in four working days or less. This represents an advantage over the previous manual procedures (176,177) and an alternative to automated assays which may be of use in some clinical and laboratory settings.

D. Radioimmunoassays:

1. The Assay

All measurements of LH in serum and pituitary extracts were made using the double-antibody procedure of Niswender et al. (169). All assays were performed in the laboratories of the Reproductive Endocrinology Program, Department of Pathology, The University of Michigan.

The assays utilized the rat pituitary extract, B-640, as the primary intra-laboratory standard for comparison. Previous bioassays (ovarian ascorbic acid depletion test, OAAD) and other radioimmunoassays have established that

B-640 has a potency of 0.03 times that of the National Institutes of Health LH standard S1 (NIH-LH-S1), i.e., each nanogram of B-640 is equivalent to 0.03 ng NIH-LH-S1. Secondary local standards consisting of pooled rat sera such as B-873 were run in each assay. The potency of these secondary standards relative to B-640 has been established by comparisons of the 50% inhibition points on plots of percentage of ^{125}I-oLH remaining bound versus log mass per tube. Even if the remaining portions of the RIA curves are nonparallel, and the samples used in generating them are thereby inferred to be dissimilar, the 50% points will yield a valid approximation of the relative potencies of the two samples (179). Such estimates gave B-873 a value of 212.0 ng B-640/ml or 6.36 ng NIH-LH-S1/ml.

There have been several (180-182) reports that the LH values measured in sera under varying physiological conditions show nonparallelism with the pituitary standard in this RIA. To ascertain that gross errors of measurement had not been introduced into the LH measurements made for the experiments to be described later, a check on the parallelism of the curves obtained with a rat pituitary extract, B-640, B-873 and a pool of rat serum generated during the studies for this thesis was performed. The percentage of ^{125}I-oLH remaining bound was plotted versus the log of the volume of sample taken for assay (which is proportional to the log of mass per tube). The curves were displaced on the plot by an arbitrary constant, α, to prevent their overlap, and were examined visually for parallelism. The rat pituitary extract and B-640 curves were markedly similar. The curve generated by B-873 was noticeably nonparallel with B-640 but was quite similar to that given by the pool of rat serum. The curve given by the pooled serum also showed some similarity to the curve generated by B-640 but, in fact, seemed not to be strictly parallel throughout its length with either the B-873 or B-640 curves. These results, shown in Figure 7, indicated the use of B-640 as the standard for pituitary extracts and B-873 as the standard for serum samples. The latter guideline was not always followed if a logit plot of ^{125}I-oLH remaining bound versus volume of unknown serum added demonstrated better parallelism with B-640 or if the value of the percentage of ^{125}I-oLH bound fell within 43-57%. In those instances direct comparison with B-640 was made. Usually, the curves of serum derived from castrated male rats appeared to resemble those from B-873 more than those from B-640; serum from intact male rats yielded curves resembling those given by B-640. Whether the direct comparison was made with B-640 or B-873 the final results for the assay were calculated in units of mass of B-640 per milliliter of unknown and are reported as such with the potency of B-640, relative to NIH-LH-S1, noted.

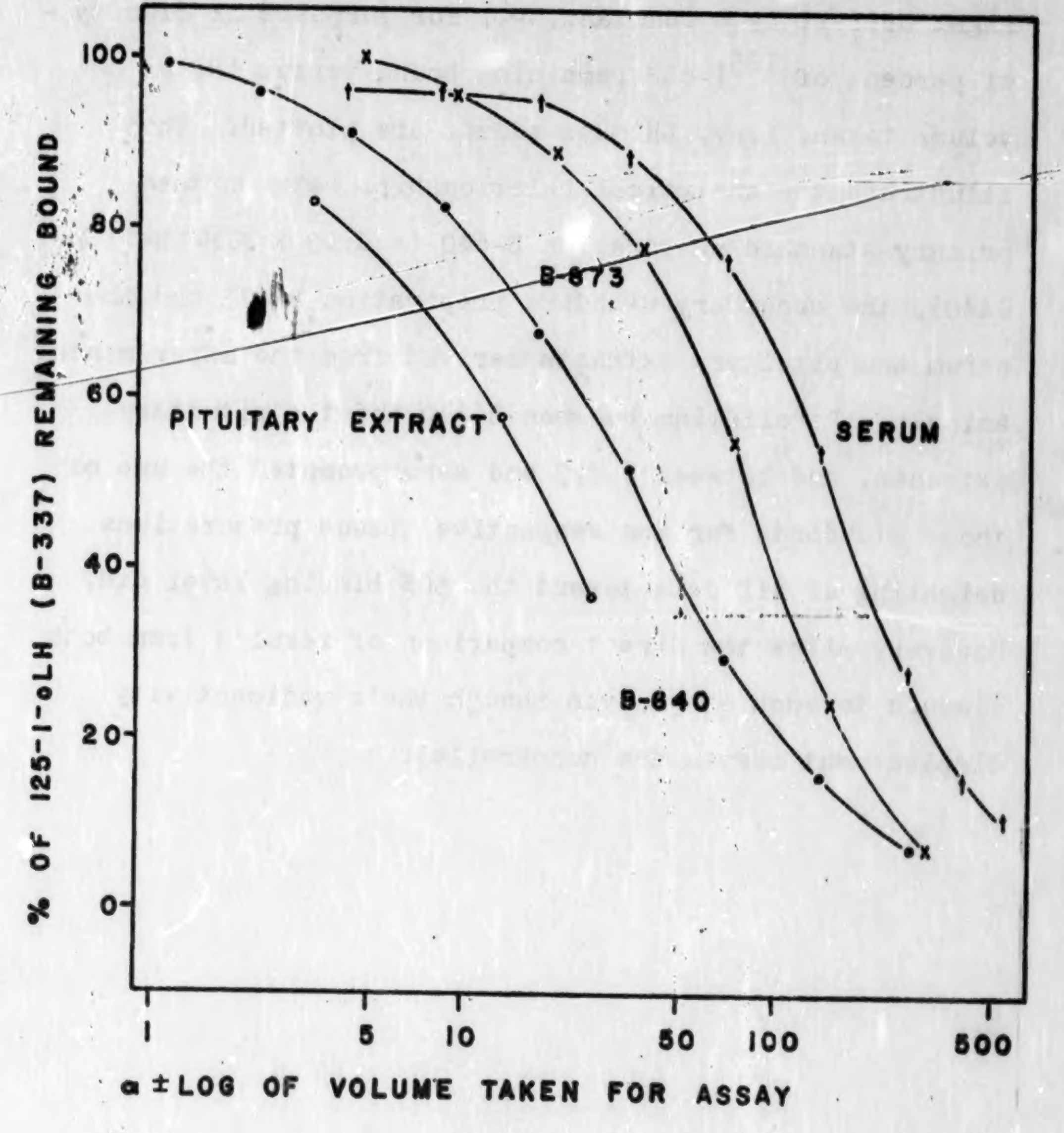
Serum for use in the LH assays was obtained either from trunk-blood, taken at the time of decapitation and

Figure 7. The Relationships Between the Response Curves for Rat Serum and Pituitary Extract and Those of Radioimmunoassay Standards B-640 and B-873

Four single representative curves - displaced to the right or left by a constant, α, for purposes of clarity - of percent of ^{125}I-oLH remaining bound versus log of volume taken, i.e., LH mass added, are plotted. They illustrate the analytical relationships between the primary standard preparation B-640 (= 0.03 x NIH-LH-S1 by OAAD), the secondary standard preparation B-873 and the serum and pituitary extracts derived from the experimental animals. Parallelism between B-640 and the pituitary extracts, and between B-873 and sera prompted the use of those standards for the respective tissue preparations. Weighting of all data toward the 50% binding level did, however, allow the direct comparison of results from both tissues to each other even though their radioactivity displacement curves are nonparallel.

FIGURE 7

THE RELATIONSHIPS BETWEEN THE RESPONSE CURVES FOR RAT SERUM AND PITUITARY EXTRACT AND THOSE OF RADIOIMMUNOASSAY STANDARDS B-640 AND B-873



prepared as discussed previously in regard to corticosterone, or from samples obtained by TVBE (see Section I. Animals). Blood obtained by TVBE was allowed to clot on ice and then centrifuged for 20 min at 5000 rpm. The serum from either bleeding procedure was decanted off after centrifugation and stored at -20°C prior to assay.

Pituitary extracts were prepared from weighed tissues either immediately after weighing or subsequent to storage of the whole tissue at -20°C. The whole pituitaries were individually homogenized in phosphate-buffered saline, pH 7.4 (PBS) (169) using six strokes of the Teflon pestle (driven by a Tri-R Stir-R Model 563C at settings of 5-8) of a Potter-Elvehjem homogenizer. The final volume of PBS diluent varied from 10 to 100 ml and was dependent upon the size of the gland and whether the rat was intact or castrated; pituitaries from castrated adults were prepared in 100 ml of PBS while those of weanling intact animals were homogenized in 10 ml of PBS. Extracts were stored at -20°C until assayed.

The volume of the duplicate sample aliquots taken for assay of LH also depended on the previous treatment of the animal; sera from intact, untreated rats were assayed by using aliquots of 200 ul; sera from long-term castrated rats or animals injected with LHRH were assayed by using aliquots of 10, 20, and/or 50 ul. The number of duplicated measurements was determined by the volume of the sample available. Single duplicates were done on sera from intact animals while three duplicates of various sizes were done on pituitary extracts.

Since the lower limit of reliability for the assay (the maximum of ^{125}I-oLH remaining bound minus two times the standard deviation of its estimation) was normally 2-10 ng of B-640/ml, values which were computed to lie below that level, although distinctly above zero, were not necessarily accurately determined. Such values are reported as they were calculated; the potential inaccuracy will be taken into account in the interpretation of experimental results. Samples which generated values which exceeded the upper limit of the standard curve of a particular assay were reassayed using smaller aliquots. The other assay variables for these assays were as follows. The mean index of precision was -0.0173, i.e., ±3.9%; the mean limit of detection (the maximum ^{125}I-oLH remaining bound minus the standard error of its estimation) was 215 ± 166 pg per assay tube; the mean sensitivity (the slope of the curve derived for the B-640 standard on the plot of percentage of ^{125}I-oLH remaining bound versus log mass of LH per tube) was -2.55 ± 0.15.

2. Effect of Sampling Time and Ether

Several authors have reported 24-hour variations in the titers of serum LH in male rats (183,184). Others have demonstrated elevations in LH levels in the serum due to ether anesthesia (36,183,185). Because the blood samples for several of my experiments were taken under ether (TVBE) and because 24-hour periodicity was briefly examined for animals treated via their o,p'-DDT-injected dams, a short control experiment was conducted. This experiment examined the effect of serial TVBE on the 24-hour periodicity of serum LH in normal adult (300-325 g) male rats.

After allowing the animals to acclimatize to the day/night cycle for four days the following sampling procedure was carried out. Beginning at 12.30 (EST) a group of 5 rats were bled under ether by tail-vein at intervals of 6 hours for 24 hours, i.e., 5 times. A second group of 15 rats was divided into 5 subgroups of 3 animals each. One of these subgroups was sacrificed by decapitation each time the ether-bled test animals were bled. Ether-bleeding and decapitation were alternated so no time bias would be introduced into the sampling for either group. All animals were maintained under normal day/night conditions except for the two short nighttime sampling periods to which the ether-bled animals were exposed. The sera which were collected at each sampling time were stored at -20°C until assayed.

The serum LH levels (± 1 standard deviation of the mean) were calculated for each group and time. These were plotted versus time of day. The individual means from the ether-bled rats were tested against the individual means of the decapitated controls by use of Student's t-tests. The values at two of the time points differ with probabilities of more than 95%; at 18.00 the ether-bled animals show serum levels approximately twice the basal levels and at midnight the decapitated controls show levels which are twice those of the ether-bled animals and twice those of the basal levels seen at any other time of day. These findings are shown in Figure 8.

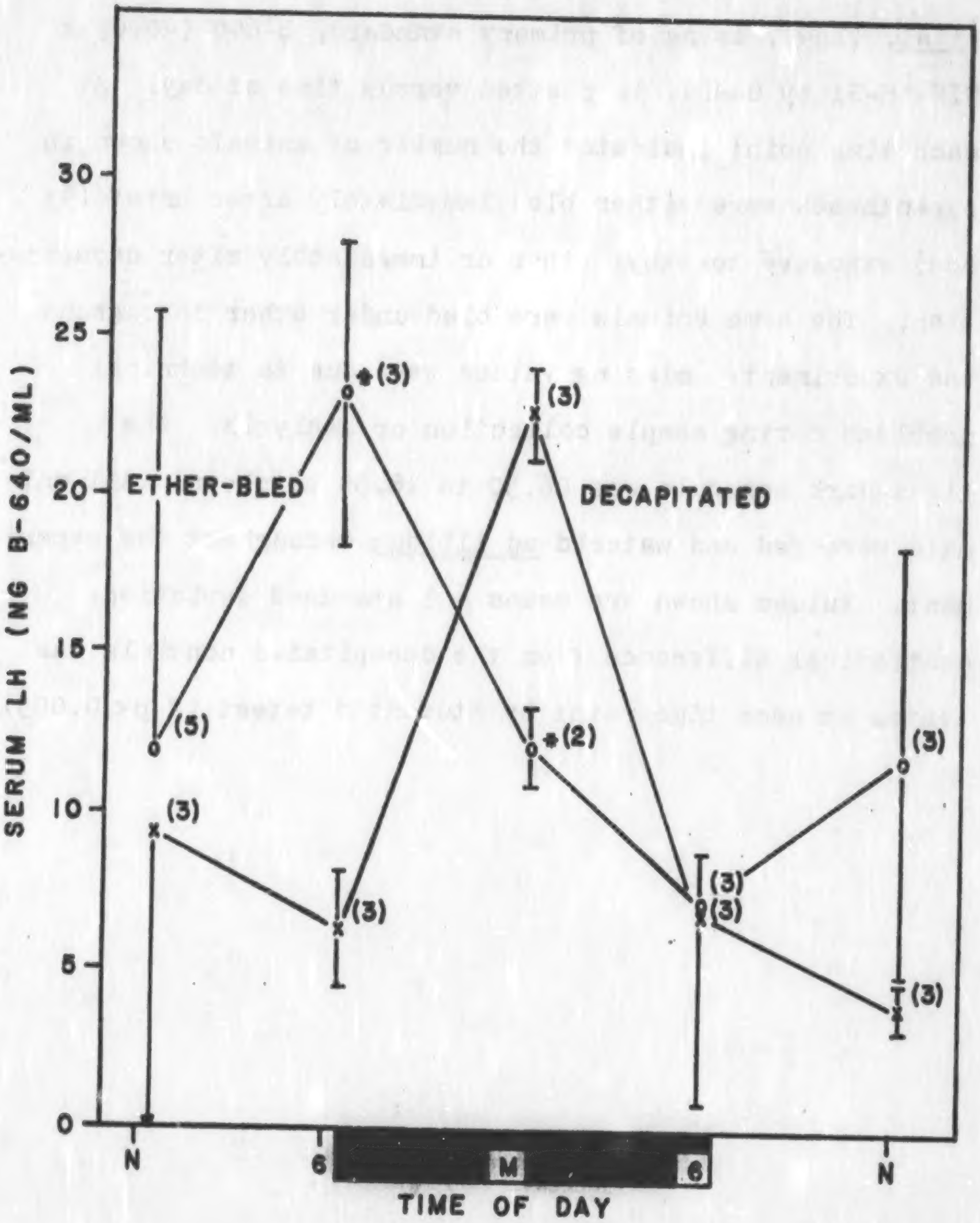
The results support those of Dunn et al. (183) and Hefco and Lackey (184) in regard to the normal 24-hour periodicity of LH in unetherized male rats. They also support the findings of Dunn et al. (183), Howland et al. (36) and Krulich and Illner (185) in regard to an ether-induced rise in serum LH during the daytime hours and the lack of such a rise at night. The plot of the 24-hour periodicity of the serum LH in ether-bled animals is nearly superimposable on similar curves generated from rats suckled by vehicle-injected dams (see intact controls, Figure 31) and other vehicle injected adult male rats. The observed pattern is also very similar to the one Lawton and Smith (186) generated using etherized male rats and an LH bioassay. Together the reported and present data indicate a variable sensitivity of the male rat hypothalamo-hypophysial axis to ether. The peak of normal LH secretion occurs during the period of peak physical activity and food intake. This period may represent a time of passive physiological resistance to stress resulting in the diminished response to ether at midnight. This low value may merely represent a spuriously low mean

Figure 8. The Effect of Repeated Tail-Vein Bleeding Under Ether on the 24-Hour Periodicity of Serum LH in Normal Adult Male Rats

Serum LH measured by the radioimmunoassay of Niswender et al. (169), as ng of primary standard, B-640 (=0.03 x NIH-LH-S1 by OAAD), is plotted versus time of day. At each time point indicated the number of animals shown in parentheses were either bled immediately after brief (45 sec) exposure to ethyl ether or immediately after decapitation. The same animals were bled under ether throughout the experiment; missing values were due to technical problems during sample collection or analysis. The light-dark schedule was 06.30 to 18.30 as shown. All animals were fed and watered ad libidum throughout the experiment. Values shown are means ± 1 standard deviation; statistical difference from the decapitated controls was tested at each time point by Student's t-test (* $p < 0.005$).

FIGURE 8

THE EFFECT OF REPEATED TAIL-VEIN BLEEDING UNDER ETHER ON THE 24-HOUR PERIODICITY OF SERUM LH IN NORMAL ADULT MALE RATS



(n = 2), however, since the similar data in Figure 31 show no difference between the serum LH levels of the intact controls near midnight and the value obtained from the unetherized control rats in the present experiments (Figure 8). If the ether-bled and control values at midnight are, in fact, similar, the data are consistent with the claim of Dunn et al. (183) that the serum LH levels cannot be elevated further at the nighttime peak due to the normal presence of an optimal LHRH output at that time. The explanation probably combines facets of both increased physical activity, respiration, etc., and a normal elevated LHRH output.

Because of the temporal positions of the serum LH elevations under normal or ether-stressed conditions, later experiments were designed to take blood samples under the most appropriate conditions. If basal levels were of interest samples were taken by decapitation near noon; whereas, if the difference between serum LH levels was to be tested in DDT treated and untreated groups, samples were taken near 18.00 (EST). In the second example the time was chosen so as to maximize any differential effect ether might have on these two groups (see also intact rats, Figure 31).

E. Estimation of o,p'-DDT Derived Residues in Rat Pups

The indirect treatment of rat pups, accomplished by allowing them to suckle o,p'-DDT-injected dams, brought up the question of administered dosage. To answer that question and to allow analyses of the insecticide used in these studies the following protocols were adapted. Neutral hydrophobic compounds similar to DDT form the bulk of the metabolites normally found in tissues (137,158,187). Therefore, the "cleanup" protocol was designed to maximize the recovery of these materials while eliminating the large bulk of contaminating, nonrelated hydrophobic compounds. It was suggested in large part by the sulfuric acid purification of hexane extracts containing PCB detailed by Widmark (188).

To measure the dam's secretion of neutral DDT metabolites into her milk and the pup's subsequent uptake of those metabolites, male pups were removed at various ages from the litters of both DDT-injected and vehicle-injected dams. The litter sizes were maintained by replacing the males removed with females of similar age. Within 10 min of removing the pups from their litters they were killed by asphyxiation with carbon dioxide. Their stomachs, containing milk curds, were then removed and the stomachs and carcasses were frozen and stored separately until the time of extraction and analysis.

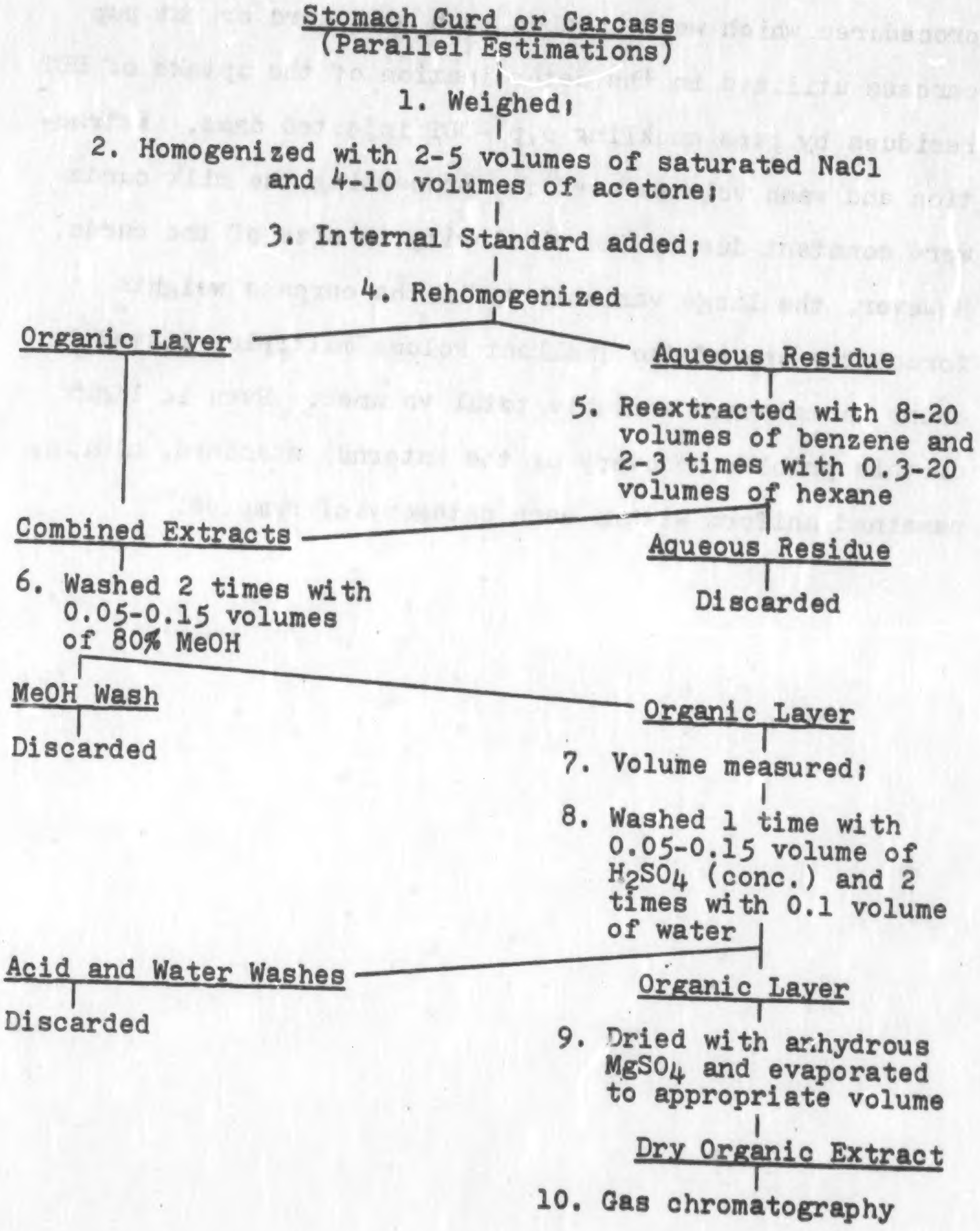
A flowchart for the "cleanup" method is shown in Figure 9. After weighing the biological sample of interest it was homogenized with 2-5 weight-volumes of saturated salt (NaCl) solution and 4-10 volumes of acetone. The homogenization was done with a Potter-Elvehjem homogenizer

Figure 9. Generalized Protocol for Estimation of DDT Analog Residues

The flowchart outlines the extraction and purification procedures which were used on each milk curd or rat pup carcass utilized in the determination of the uptake of DDT residues by pups suckling o,p'-DDT injected dams. Extraction and wash volumes used for processing the milk curds were constant due to the similarity in size of the curds. However, the large variability in the carcass weights forced the use of the smallest volume multiples listed in order to maintain workable total volumes. Even in light of this problem recovery of the internal standard, aldrin, remained uniform within each category of samples.

Figure 9

Generalized Protocol for Estimation of DDT Analog Residues


Stomach Curd or Carcass
(Parallel Estimations)
1. Weighed;
2. Homogenized with 2-5 volumes of saturated NaCl and 4-10 volumes of acetone;
3. Internal Standard added;
4. Rehomogenized
Organic Layer
Aqueous Residue
5. Reextracted with 8-20 volumes of benzene and 2-3 times with 0.3-20 volumes of hexane
Combined Extracts
Aqueous Residue
Discarded
6. Washed 2 times with 0.05-0.15 volumes of 80% MeOH
MeOH Wash
Discarded
Organic Layer
7. Volume measured;
8. Washed 1 time with 0.05-0.15 volume of H2SO4 (conc.) and 2 times with 0.1 volume of water
Acid and Water Washes
Discarded
Organic Layer
9. Dried with anhydrous MgSO4 and evaporated to appropriate volume
Dry Organic Extract
10. Gas chromatography

at high speed if the curd was being analyzed or with a Waring blendor equipped with an explosion-proof chamber if whole pups were being extracted. After the initial homogenization a known quantity of the internal chromatography standards used for pesticide analysis, aldrin and p,p'-DDT, were added (0.5 ug of each were added in curd extractions; 50 ug of each were added in whole-body extractions). The solution was then rehomogenized and placed either into a centrifuge tube or a separatory funnel. When the organic and aqueous phases had settled or been separated by centrifugation the organic layer was removed and saved. The aqueous residue was reextracted once with 8-20 volumes of benzene and 2 or 3 times with 0.3-20 volumes of hexane. The aqueous residue was discarded. All the organic phases were combined and washed twice with 0.05-0.15 volume of 80% aqueous methanol. The alcoholic washes were discarded and the volume of the organic layer measured. This solution was then washed with 0.05-0.15 volume of concentrated H_2SO_4. Following the acid wash the organic layer was washed twice with 0.1 volume of water. Acid and water washes were both discarded. This step, 8, removed the large majority of the hydrophobic impurities left in the solution. The wet organic phase was dried over anhydrous $MgSO_4$ and then evaporated on a steam-cone under nitrogen to near 5 ml (curd samples) or 50 ml (whole pups). Final volumetric adjustments were made by adding hexane. Aliquots of 1-8 ul of these dry extracts were injected directly into the gas chromatograph for qualitative and quantitative analyses.

The precise volumes used in extractions and washes varied with sample weight. When dealing with whole body residues a ceiling of 300 ml was used for the addition of benzene and 600 ml for a total extraction volume. Other extraction and wash volumes were scaled up or down within the limits shown to prevent the sample preparation from becoming unwieldy. For small to moderate size samples, however, the upper limits of the volumes shown were favored to minimize problems with emulsions and to maximize clean recoveries. For curd samples standardized volumes were used throughout the preparation: 5 ml of saturated salt solution and 10 ml of acetone, 20 ml of benzene and 2 times 20 ml hexane, 2 times 10 ml of 80% methanol, 10 ml concentrated H_2SO_4, 10 and 5 ml of water. When calibration standards were processed by this procedure all steps followed those used for the preparation of stomach curd samples.

All concentrated extracts were analyzed by a Varian 2100 GC system which incorporated a ^{3}H-Scandium foil electron capture detector. The chromatography was performed using a 180 cm x 2 mm glass column packed with 80/100 mesh Gas Chrom Q coated with 6% QF-1 and 4% SE-30. Nitrogen carrier gas was used at a flow rate of 63 ml/min under a pressure head of 70 psi. The injector temperature was 245°C; the detector was held at 295°C. Chromatograms were run under a shallow temperature program, 175° to 195°C at 0.5°/min, which had been demonstrated not to interfere with peak resolution or baseline stability. Attenuator settings varied slightly but were usually at 16×10^{-10} amp/mv; detector standing current was normally 2×10^{-9} amp. Chart speed was 0.5 cm/min. The column packing was suggested by the work of McCully and McKinley (189) and Abou-Donia and Menzel (190); the retention times observed agree fully with their findings. Relative retention times with respect to aldrin were: 1.45, o,p'-DDE; 1.84, p,p'-DDE; 2.04, o,p'-DDD; 2.39, o,p'-DDT; 2.57, p,p'-DDD; 3.06, p,p'-DDT. Three typical chromatograms are shown in Figure 10.

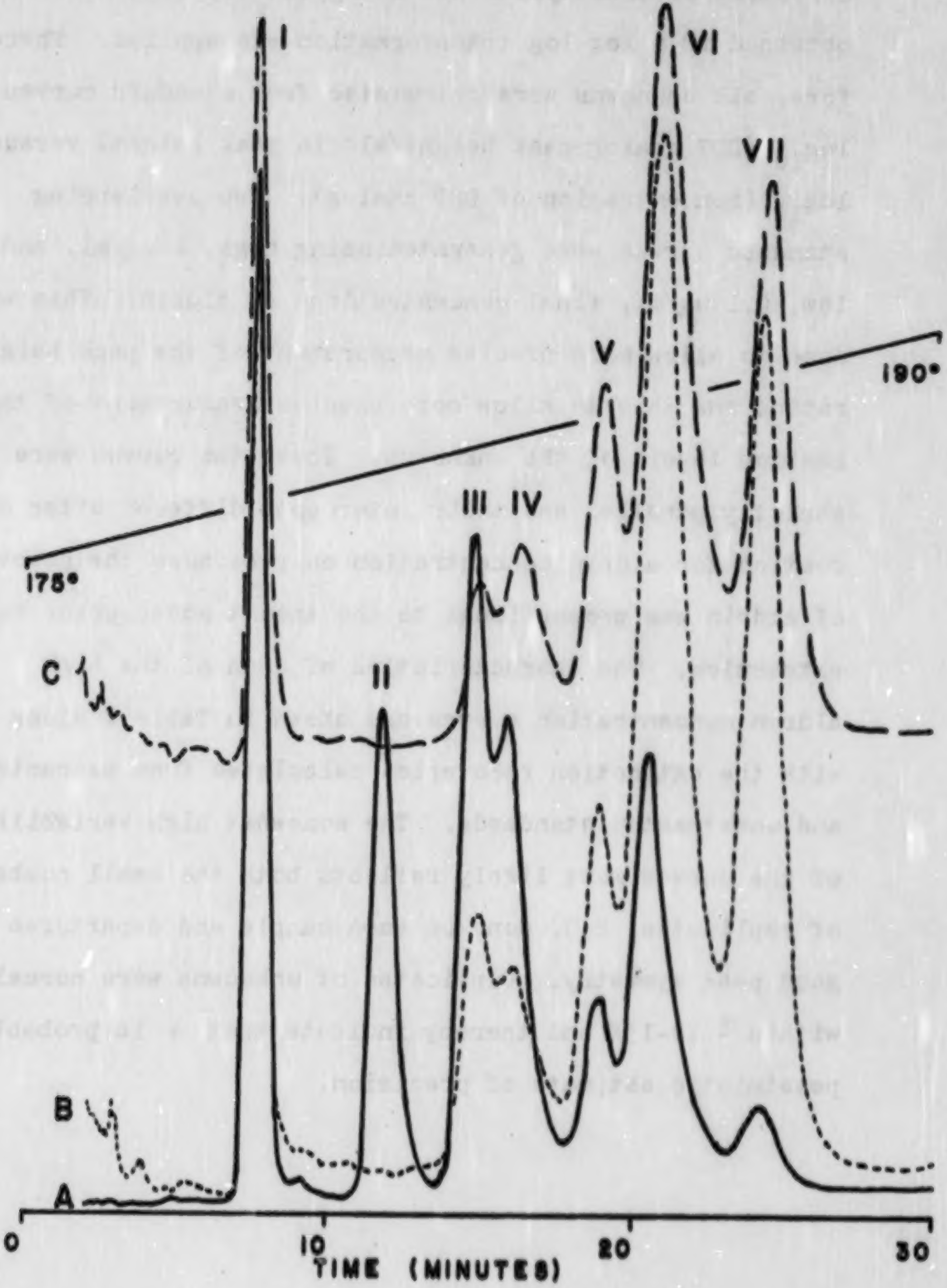
Since the o,p'-DDT was contaminated with 1.5% p,p'-DDT (see II. Chemicals) the knowledge that p,p'-DDT is biologically stored preferentially to o,p'-DDT (166) militated against any reliance on the use of p,p'-DDT as an internal standard. Therefore, all analyses were performed using only aldrin as the standard for internal comparison and deleted quantitation of o,p'-DDT. The sharpness and form of most of the peaks in the chromatogram indicated that plots of peak height versus mass, or some transformation of these measurements, would be suitable for quantitation. Calibration curves were generated by chromatographing mixtures containing various amounts of o,p'-DDE, p,p'-DDE, o,p'-DDD, o,p'-DDT and p,p'-DDD both before and after extraction. The linearity of the curves of DDT analog

Figure 10. Typical Gas Chromatograms Obtained During Analyses of o,p'-DDT and Its Analogs

Profiles of detector response versus retention time are shown for 3 typical samples. Sample A, ——, is a 1.2 ul injection of an unextracted standard solution containing 1.5 ng/ul of each of o,p'-DDE (II), p,p'-DDE (III), o,p'-DDD (IV), o,p'-DDT (V), p,p'-DDD (VI) and 1.0 ng/ul of both aldrin (I) (internal standard) and p,p'-DDT (VII). Sample B, ---, is a 2.2 ul injection of the residues extracted from a 15 day old rat pup which had nursed an o,p'-DDT injected dam; 1.0 ng/ul of internal standard has been added. Sample C, – –, is a 1.75 ul injection of the hexane extract of a milk curd from the stomach of a 10 day old rat pup which had nursed an o,p'-DDT injected dam; 0.1 ng/ul of internal standard has been added.

All samples were run on a 2 m x 2 mm column of 6%/4% QF-1/SE-30 on 80/100 mesh Gas Chrom Q under a temperature program of 0.5°/min from 175° to 195°C. Injector and detector temperatures were 245° and 295°C, respectively. Gas, N_2, flow rates were 63 ml/min under a pressure head of 70 psi. An electron capture detector was used at an attenuator setting of 16×10^{-10} amp/mv; chart speed was 0.5 cm/min.

FIGURE 10
TYPICAL GAS CHROMATOGRAMS OBTAINED DURING ANALYSES OF o,p'-DDT AND ITS ANALOGS



peak height/aldrin peak height versus DDT analog concentration was tested by least-squares regression. Though the curvature of these plots was not great the best lines were obtained if a log-log transformation was applied. Therefore, all unknowns were calculated from standard curves of $\log_{10}$ (DDT analog peak height/aldrin peak height) versus $\log_{10}$ (concentration of DDT analog). Two overlapping standard curves were generated using high, 1 ug/ml, and low, 0.1 ug/ml, final concentrations of aldrin. This was done to allow more precise measurement of the peak height ratios and thus to allow more precise measurement of the residue levels of the unknowns. These two curves were strictly parallel and their intercepts differed after correction for aldrin concentration only because the recovery of aldrin was proportional to the amount added prior to extraction. The characteristics of each of the high aldrin concentration curves are shown in Table 2 along with the extraction recoveries calculated from extracted and unextracted standards. The somewhat high variability of the curves most likely reflects both the small number of replicates, 2-3, done on each sample and departures from good peak symmetry. Duplicates of unknowns were normally within ± 10-15% and thereby indicate that λ is probably a pessimistic estimate of precision.

Table 2

Standard Curves for Determination of DDT Analogs by Electron-Capture Gas Chromatography

Variable	Analog				
	o,p'-DDT	o,p'-DDD	o,p'-DDE	p,p'-DDD	p,p'-DDE
Slope[1] b±SD	0.986±0.032	0.846±0.024	0.876±0.021	0.488±0.029	0.836±0.024
Intercept[2] a±SD	-0.781±0.020	-0.434±0.017	-0.469±0.014	-0.324±0.017	-0.346±0.017
Correlation Coefficient R	0.994	0.993	0.995	0.975	0.993
SE_y[3]	0.065	0.068	0.061	0.065	0.070
λ[3]	0.066	0.080	0.070	0.133	0.084
Useful Range[4] (ug/ml)	0.008-9.0	0.009-9.0	0.019-9.0	0.012-9.0	0.007-9.0
% Recovery[5]	97.2	106.3	102.8	103.0	102.7

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[1] The standard curve is a plot of Log_{10} (Peak Height Analog/Peak Height Aldrin) versus Log_{10} (Analog Concentration) and is of the form Log y = a + b Log x.

[2] The intercept is given for the standard curves used at higher analog concentrations which utilized an aldrin (internal standard) concentration of 1 ug/ml; at lower analog concentrations an aldrin standard of 0.1 ug/ml was used and the intercept increased.

Table 2 (Cont.)

---

[3] The error in the predictability of Log y given Log x is the standard error of the estimate, SE_y; the error in the predictability of Log x given Log y is the index of precision, λ, and equals SE_y/b.

[4] The useful range is calculated from the linear portion of the parallel curves generated for both high and low concentrations of analogs.

[5] Recovery of extracted versus nonextracted standards; aldrin recovery was 66% at 0.1 ug/ml and 84% at 1 ug/ml.

F. Testicular Incubations:

1. Protocol

Measurements were undertaken to investigate the existence, and possible site, of subcellular interactions between o,p'-DDT and the testicular steroids. Incubations of $[7\ n\text{-}^{3}H]\text{-}\Delta^{5}$-pregnenolone were conducted in the presence or absence of o,p'-DDT in testicular homogenates. The protocol, described below, was so designed that it also showed the presence or absence of o,p'-DDT metabolism in the same homogenates.

Testes, obtained during castrations or after animal sacrifice, were weighed, decapsulated and homogenized at 4°C in 6 weight-volumes of Buffer I. Buffer I is a modified Ringer's solution suggested by Moldeus et al. (191) which resembles buffers previously used both in steroid incubations with testes (182,193) and in drug incubation with hepatic microsomes (191). Buffer I is 125 mM NaCl, 6 mM KCl, 5 mM $MgCl_2$, 15 mM Na_2HPO_4, 10 mM succinate adjusted to pH 7.4. The homogenization was achieved with 6 slow strokes of a Potter-Elvehjem homogenizer fitted with a Teflon pestle (driven by a Tri-R Stir-R Model 563C at settings of 5-8). The homogenate volume was measured and poured into 50 ml centrifuge tubes. These were centrifuged at 4°C at 500 x g for 10 minutes in a Lourdes model LRA centrifuge. After centrifugation the supernatants were decanted into flasks and held on ice prior to incubation.

Incubations were performed in 20 ml beakers to which 200 ul of propylene glycol, measured amounts of ethanol or ethanolic solutions of Δ^5-pregnenolone and/or o,p'-DDT, cofactors and 1.90 ml of Buffer I were added. The cofactors included an NADPH-generating system and NADH; their concentrations in the final incubation volume were 0.825 mM $NADP^+$, 0.4125 mM G-6-P, 1 unit of G-6-P DH/ml and 0.4125 mM NADH. Substrate concentrations were 1 nmole/ml in the final incubation volume for both Δ^5-pregnenolone (including 5 uCi of $[7\ n\text{-}^3H]$-Δ^5-pregnenolone) and o,p'-DDT. Incubations were initiated by vigorously adding 4.0 ml of supernatant (40-60 mg of protein) or Buffer I to the chilled cofactor-substrate solution. Immediately after this addition a 1.0 ml aliquot was removed for a zero-time determination of steroids and/or DDT metabolites. The remaining solution was quickly placed into a Dubnoff metabolic shaker set at 20 rpm. The incubation proceeded under air at 37°C. At 5, 10, 30 and 60 minutes of incubation additional 1.0 ml aliquots were taken. After incubation the residual volume of solution was retained for determination of protein and to check the number of tritium counts initially added to the incubation. Protein was measured by the Biuret method (194) using BSA as a standard. Counts were determined on a 0.1 ml aliquot which was allowed to air dry prior to addition of scintillation fluid. The protocol is summarized in Table 3.

Each of the incubations, 1-5, was designed to probe a different facet of the potential differences between

Table 3

Incubations Performed[1]

	Volume of Solutions Added to Each Flask				
Addition	Incubation Number				
	1	2	3	4	5
Buffer I[2] (ml)	1.90	1.90	1.90	1.90	5.90
Cofactors[3]	+	+	+	+	+
Propylene Glycol (ul)	200	200	200	200	200
Supernatant[4] (ml)	4.00	4.00	4.00	4.00	0
[7 n-^{3}H]-Δ^5-Pregnenolone[5] (ul)	5	5	0	0	5
cold Δ^5-Pregnenolone[6] (ul)	5	5	0	0	5
o,p'-DDT[7] (ul)	8.2	0	8.2	0	0
Ethanol (ul)	0	8.2	10.0	18.2	8.2
Total Volume (ml)	6.20	6.20	6.20	6.20	6.20

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[1] Flasks were incubated in a Dubnoff metabolic shaker at 20 rpm under air at 37°C and samples taken at 0, 5, 10, 30 and 60 minutes of incubation. The residual volume of each flask was retained for determination of protein by the Biuret procedure (BSA standard) and for determination of the total initial content of ^{3}H counts.

[2] Buffer I is 125 mM NaCl, 6 mM KCl, 5 mM $MgCl_2$, 15 mM Na_2HPO_4, 10 mM succinicate at pH 7.4.

Table 3 (Cont.)

---

[3] Final cofactor concentrations in the total incubation volume were 0.825 mM NADP$^+$, 0.4125 mM G-6-P, 0.4125 mM NADH and 1 unit of G-6-P DH/ml.

[4] The 500 x *g* supernatant of a 6:1 homogenate of testicular tissue in Buffer I was used.

[5] Concentration of the ethanol solution added was 1 uCi/ul.

[6] Concentration of the ethanol solution added was 1.18 nmoles/ul.

[7] Concentration of the ethanol solution added was 0.75 nmole/ul.

supernatants derived from treated and untreated animals. Number 5 serves as a control for thermal or nonenzymatic steroid transformations and therefore as the baseline for steroid metabolism in supernatants from both treated and untreated animals. Number 4 provides a control for the endogenous presence and/or conversion of o,p'-DDT. Number 3 allows the measurement of the metabolism of DDT in the absence of exogenously added steroid while number 2 allows the measurement of the metabolism of Δ^5-pregnenolone in the absence of exogenously added o,p'-DDT. Number 1 provides a direct test of the interactions of steroid metabolism and DDT metabolism by allowing simultaneous measurement of both sets of metabolites.

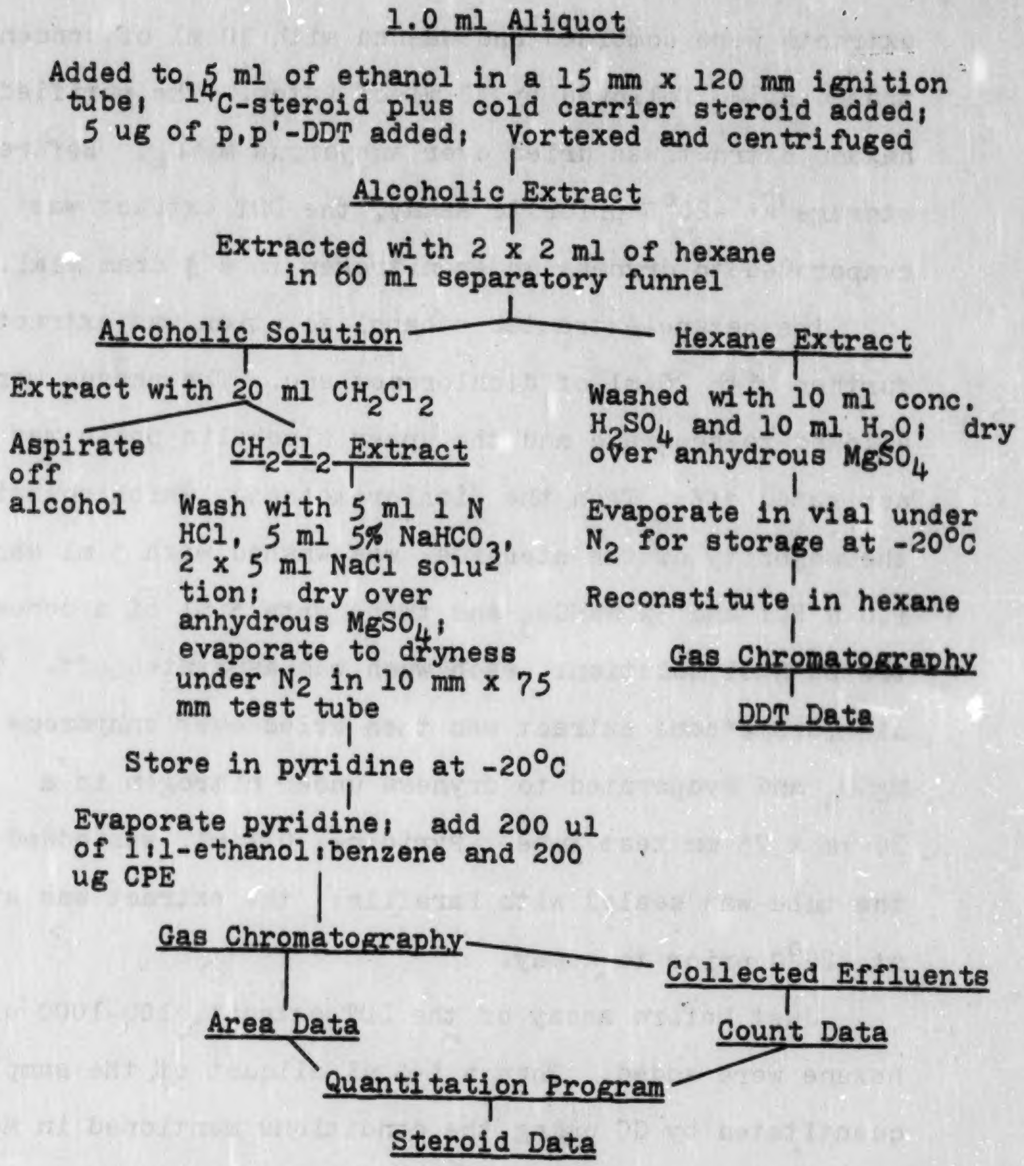
To allow this simultaneous measurement of both sets of metabolites an extraction and purification scheme was devised; it is summarized in Figure 11. The aliquots taken during incubation were forcefully expressed into 5 ml of ethanol held in a 15 mm x 120 mm ignition tube. This stopped any metabolic conversions and began the extraction process. If further processing was not begun immediately the solutions were stored at -20°C. A recovery marker (60,000 dpm ^{14}C-steroid) and carrier steroid (100 ug) were then added for each of the several steroids of interest. An internal standard, 5 ug of p,p'-DDT, was also added to monitor the recovery of DDT metabolites. This solution was Vortexed thoroughly and then centrifuged for 10-15 minutes at top speed in a Clay-Adams tabletop

Figure 11. Protocol for Extraction and Partial Purification of DDT and Steroid Metabolites in *in vitro* Incubations

The 1.0 ml samples taken from the *in vitro* incubations carried out under conditions described in Table 3 were prepared for analysis by this procedure. Steroids added to aid in recovery and estimation included 100 ug cold steroid plus 60,000 dpm 4-^{14}C-steroid each for: Δ^5-pregnenolone, progesterone, 17α-hydroxyprogesterone, dehydroepiandrosterone, Δ^4-androstenedione, and tesosterone. The p,p'-isomer of DDT was also added (5 ug) as an internal standard and recovery tracer for the DDT analogs. The internal standard for the steroids, cholesterol-3-propyl ether (CPE), was added just prior to gas chromatography in a system which split the column effluent in approximately a 3:1 ratio between a collection capillary and the mass detector of the chromatograph. Specifics of the measurements are discussed in Methods Section III.F.2. and in Chapter 6.

Figure 11

Protocol for the Extraction and Partial Purification of DDT and Steroid Metabolites in *in vitro* Incubations



1.0 ml Aliquot

Added to 5 ml of ethanol in a 15 mm x 120 mm ignition tube; ^{14}C-steroid plus cold carrier steroid added; 5 ug of p,p'-DDT added; Vortexed and centrifuged

Alcoholic Extract

Extracted with 2 x 2 ml of hexane in 60 ml separatory funnel

Alcoholic Solution

Extract with 20 ml CH_2Cl_2

Aspirate off alcohol

CH_2Cl_2 Extract

Wash with 5 ml 1 N HCl, 5 ml 5% $NaHCO_3$, 2 x 5 ml NaCl solution; dry over anhydrous $MgSO_4$; evaporate to dryness under N_2 in 10 mm x 75 mm test tube

Store in pyridine at -20°C

Evaporate pyridine; add 200 ul of 1:1-ethanol:benzene and 200 ug CPE

Gas Chromatography

Area Data

Collected Effluents

Count Data

Quantitation Program

Steroid Data

Hexane Extract

Washed with 10 ml conc. H_2SO_4 and 10 ml H_2O; dry over anhydrous $MgSO_4$

Evaporate in vial under N_2 for storage at -20°C

Reconstitute in hexane

Gas Chromatography

DDT Data

centrifuge. The extract was carefully decanted off into a 60 ml separatory funnel. The pellet was reextracted with another 2 ml of ethanol. The combined ethanol extracts were extracted twice with 2 ml of hexane. These hexane extracts were combined and washed with 10 ml of concentrated H_2SO_4 followed by 10 ml of water. The purified hexane extract was dried over anhydrous $MgSO_4$. Before storage at -20°C prior to assay, the DDT extract was evaporated to dryness under nitrogen in a ½ dram vial.

The hexane-extracted ethanol solution was extracted further with 20 ml of dichloromethane. The phases were allowed to separate and the upper alcoholic phase was aspirated off. Then the dichloromethane, which contained the majority of the steroids, was washed with 5 ml each of 1.0 N HCl and 5% $NaHCO_3$ and twice with 5 ml of a concentrated NaCl solution; each wash was aspirated off. The dichloromethane extract was then dried over anhydrous $MgSO_4$ and evaporated to dryness under nitrogen in a 10 mm x 75 mm test tube. Pyridine, 0.1 ml, was added and the tube was sealed with Parafilm; the extract was stored at -20°C prior to assay.

Just before assay of the DDT extract, 100-1000 ul of hexane were added. Then a 1-5 ul aliquot of the sample was quantitated by GC using the conditions mentioned in Methods Section E., Estimation of o,p'-DDT Derived Residues in Rat Pups. Before assay of the steroid extract the pyridine was evaporated off with nitrogen and 200 ul of a 1:1 solution of benzene and ethanol containing 200 ug of the chosen internal standard for GC of steroids, cholesterol-3-propyl ether (CPE), was added to make up the assay solution.

2. Estimation of Steroids by Gas Chromatography and Scintillation Counting

Aliquots of the final steroid assay solution, 1-5 ul, were injected onto a 350 cm x 2 mm glass column equipped with an effluent splitter. The column was packed with 80/120 mesh Gas Chrom Q coated with 1.5% OV-7 and 1.5% SE-54. The effluent splitter favored an effluent-capture-tube over the flame-ionization detector by 3 to 1, i.e., 75% of the effluent was captured and 25% was burned for qualitative and quantitative analysis. Chromatograms were run using nitrogen as the carrier gas at a flow rate of 25 ml/min (head pressure was 70 psi); hydrogen and oxygen were used as the flame support gases at flow rates of 30 and 60 ml/min, respectively. Injector and detector temperatures were 250° and 295°C, respectively. Column temperature was programmed on a linear gradient of 1°/min from 175° to 275°C. Chart speed was 10 cm/hour. Two sample chromatograms are shown in Figure 12.

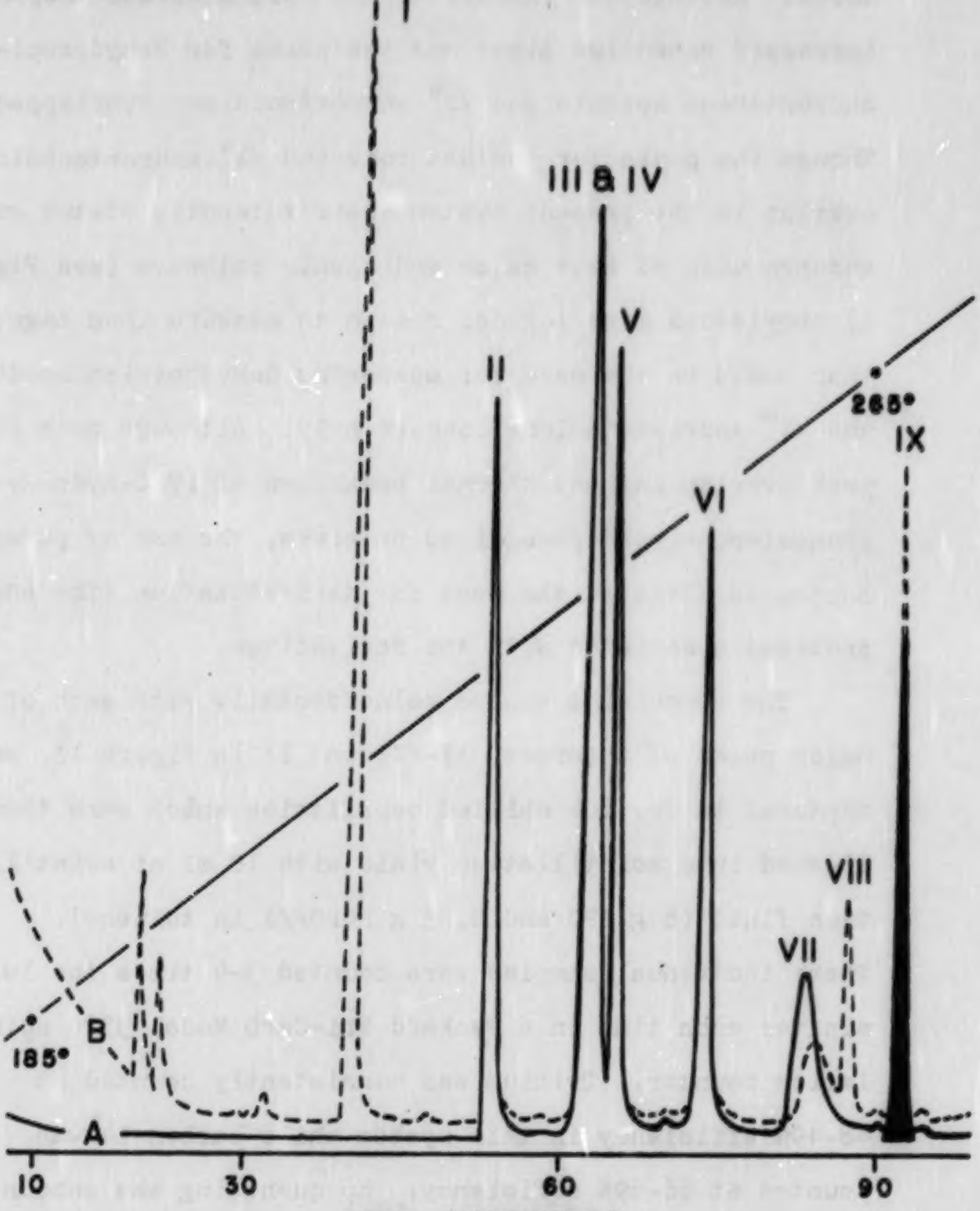
The use of acetate and methyloxime-silyl derivatives (195) on this and several other columns had been explored with varying degrees of success, in terms of efficient effluent capture and adequate resolution of the main steroid peaks of interest. Methyloxime-silyl derivatives were chromatographically more stable and volatile than the

Figure 12. Typical Gas Chromatograms Obtained During Analysis of Testicular Δ^5-Pregnenolone Metabolites

Two sample chromatograms obtained during analyses of testicular metabolites of Δ^5-pregnenolone are shown. Plot A was taken from a 7.75 ul injection of a calibration standard containing 2.0 ug/ul each of dehydroepiandrosterone (II), Δ^4-androstenedione (III), testosterone (IV), Δ^5-pregnenolone (V), progesterone (VI), 17α-hydroxyprogesterone (VII) and 200 ng/ul cholesterol-3-propyl ether (internal standard) (IX). Plot B was obtained from a 6.05 ul injection of an extract prepared via the protocol of Figure 10 from a sample taken at 30 minutes of incubation of a testicular homogenate. The homogenate was prepared from the testes of an adult male rat which had been injected daily on days 1-5 of age with 0.05 ml of sesame oil containing 40 ug estradiol-17-valerinate. In addition to the peaks II-VII and IX indicated in the calibration mixture two unidentified peaks, I and VIII, are also evident. It should be noted that in plot B the bulk of the steroid mass responsible for peaks II-VII is due to the added carrier steroids; the similarity of the inter-peak relationships between Plots A and B is a reflection of the similarity of the inter-steroid mass ratios in the standard solution and the unknown solution containing the added carrier steroids. See Materials and Methods Section IV.F.2. for chromatographic details.

FIGURE 12

TYPICAL GAS CHROMATOGRAMS OBTAINED DURING ANALYSES OF TESTICULAR Δ^5-PREGNENOLONE METABOLITES



parent compounds but their capture efficiency was poor due to their tendency to form stable aerosols when cooled (195) rather than condensing onto the capillary capture tubes. Acetate derivatives worked well also but they had increased retention times and the peaks for dehydroepiandrosterone acetate and Δ^4-androstenedione overlapped. Though the peaks for testosterone and Δ^4-androstenedione overlap in the present system their metabolic status as end-products of both major androgenic pathways (see Figure 1) provides a more logical reason to measure them together than would be the case for measuring dehydroepiandrosterone and Δ^4-androstenedione concurrently. Although both this peak overlap and the thermal breakdown of 17α-hydroxyprogesterone were recognized problems, the use of parent compounds obviated the need for derivatization time and the problems associated with the derivatives.

The components eluted coincidentally with each of the major peaks of interest, II-VII and IX in Figure 12, were captured in dry-ice chilled capillaries which were then flushed into scintillation vials with 10 ml of scintillation fluid (6 g PPO and 0.25 g POPOP/l in toluene). These individual samples were counted 3-4 times for 10 minutes each time in a Packard Tri-Carb Model 3320 scintillation counter. Tritium was consistently counted at 48-49% efficiency in this system while carbon-14 was counted at 66-69% efficiency; no quenching was encountered. Counts were converted to ^{3}H and ^{14}C dpm by using a program written for a Wang 614 calculator which was based on the discriminator-ratio method of Okita et al. (196). Counting errors were normally less than 1.2% for ^3H and less than 1.4% for ^{14}C.

Initial qualitative identification of the peaks was based on the retention times relative to CPE of known steroids. Solutions of various known concentrations of dehydroepiandrosterone, Δ^4-androstenedione, testosterone, Δ^5-pregnenolone, progesterone and 17α-hydroxyprogesterone, each of which contained 1 ug/ul of CPE, were chromatographed to identify the various peaks and to calibrate the system for the later quantitation of unknowns. The retention times relative to CPE were: 0.57, dehydroepiandrosterone; 0.67, Δ^4-androstenedione and testosterone; 0.69, Δ^5-pregnenolone; 0.79, progesterone; and, 0.91, 17α-hydroxyprogesterone. Peak area data for these chromatograms were calculated both by triangulation and by an automatic integration system incorporating an Adams-Smith interface and an experimental program written for a Wang 614 calculator; the results from both methods compared favorably for all but low, broad peaks which the automatic system failed to detect. The logarithms of peak areas, normalized by the peak area of CPE, were plotted versus the log of the concentration of steroid injected. These standard calibration curves along with the mean percentage of steroid captured for counting are described in Table 4. Overall recoveries for the steroids, including

Table 4

Standard Curves for Determination of Steroids by Gas Chromatography

Variable	Steroid[1]				
	DHA	Δ^4 + T	Δ^5	P	17α
Slope[2] b±SD	1.005±0.028	0.988±0.024	1.047±0.031	1.004±0.031	0.994±0.025
Intercept[2] a±SD	0.183±0.018	0.440±0.013	0.153±0.020	0.086±0.019	-0.023±0.018
Correlation Coefficient R	0.993	0.995	0.992	0.992	0.995
SE_y[3]	0.066	0.049	0.073	0.072	0.061
λ[3]	0.066	0.050	0.070	0.072	0.061
Useful Range[4] (ug/ml)	0.02-25	0.04-50	0.02-25	0.02-25	0.02-25
% Recovery[5]	68.6	59.0	88.5	78.1	49.1

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[1] DHA, dehydroepiandrosterone; Δ^4, Δ^4-androstenedione; T, testosterone; Δ^5, Δ^5-pregnenolone; P, progesterone; 17α, 17α-hydroxyprogesterone.

[2] The standard curve is a plot of Log_{10}(Peak Area:Steroid/Peak Area:Cholesterol-3-propyl ether) versus Log_{10}(Steroid Concentration) and is of the form Log y = a + b Log x.

[3] The error in the predictability of Log y given Log x is the standard error of the estimate, SE_y; the error in the predictability of Log x given Log y is the index of precision, λ, and equals SE_y/b.

Table 4 (Cont.)

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[4] The least concentration detectibly greater than zero + 2 λ to the maximum measured.

[5] Recovery of ^{14}C-steroids injected onto the column and trapped with a chilled capillary tube divided by the actual fraction of chromatographic effluent flowing to the capillary.

extraction, GC and capillary recovery (which is shown in Table 4) ranged from 37% for 17α-hydroxyprogesterone to 66% for Δ^5-pregnenolone. Due to the somewhat high values of λ all measurements on unknowns were done at least in duplicate.

To further support the identities of the steroids with the peaks involved in the chromatograms, $^3H/^{14}C$ ratios were generated for each GC peak of interest both before and after each of a series of further purification steps. Peak effluents were combined from 5 chromatograms of an assay sample generated from the aliquot taken after 60 minutes of incubation of a testicular supernatant from a sesame oil-injected rat. The combined effluents for each peak were eluted from the capillaries with 3 ml of acetone and evaporated to dryness. They were taken up in 200 ul of ethyl ether and 10 ul were counted. Fifty micrograms of each of Δ^4-androstenedione and testosterone were added to the solution containing the effluent of peak III and IV (Figure 12). This solution was applied to a TLC plate and run twice in 7:3-hexane:acetone. The spots were located under UV light, scraped and then eluted with 1.5 ml 1:1-methanol:chloroform. Aliquots of these eluents were counted.

After the addition of 5 mg of the putative steroid each peak effluent was submitted to derivatization with either acetic anhydride or methoxylamine hydrochloride. GC effluents corresponding to peaks II, IV, V and VII were acetylated while the methyloximes were formed for those corresponding to peaks II and VI. After derivatization the steroids were recrystallized from ethanol/water. The crystals were collected and dissolved in acetone; aliquots were again counted. Recrystallization of the derivatives was carried out twice more, first from acetone/water and then from ethyl ether/hexane. In both cases aliquots of the collected crystals were counted. The $^3H/^{14}C$ ratios obtained are given in Table 5. Most of the steroids initially lose some ^3H counts before the ratio stabilizes, indicating the inclusion of some other labelled metabolites within the captured effluent or the inclusion of column-derived background counts for which corrections have not been made. (Based on effluents corresponding to the unlabelled CPE peak, column effluents include nonspecific counts amounting to 1-2% of the total number of counts injected onto the column for a particular chromatogram for both ^3H and ^{14}C.) The peak containing Δ^4-androstenedione and testosterone appears reasonably pure as does 17α-hydroxyprogesterone. The probable tailing of testosterone into the Δ^5-pregnenolone peak explains the initial loss of ^3H for this peak. The progesterone peak obviously contains at least one other product and the dehydroepiandrosterone peak appears to be grossly contaminated by another product or products. Both these last peaks do, however, show an approach to a stabilized $^3H/^{14}C$ ratio by the third crystallization and therefore

Table 5

$^{3}H/^{14}C$ Ratios[1]: Evidence Supporting Most of the Steroid Identities Assigned to the Gas Chromatographic Peaks Obtained During Analysis of Steroid Metabolite Mixtures

Treatment	Steroid[2] and Assigned Peak					
	DHA/II	Δ^{4}/III	T/IV	Δ^{5}/V	P/VI	17α/VII
Captured Peak	4.333	----- 9.700------		1.125	1.189	1.190
TLC (7:3-Hexane:Acetone)	-----	3.076 ----- 9.259------	15.766	-----	-----	-----
Derivatization[3] and Crystallization I (EtOH/Water)	2.763	2.980 -----10.397------	19.369	0.372	0.672	0.953
Crystallization II (Acetone/water)	1.790	2.770 -----11.174------	20.168	0.186	0.506	0.958
Crystallization III (Ethyl Ether/Hexane)	1.398	3.130 -----10.583------	20.118	0.219	0.484	0.937

[1] Five injections (34.6 ul) of an extract from a sample taken after 60 minutes of incubation of a testicular homogenate with [7n-^{3}H]-Δ^{5}-pregnenolone and to which ^{14}C-carrier steroids had been added were submitted to gas chromatography; the indicated peaks (Figure 32) being trapped and analyzed. All ratios represent single determinations; 40 minute counts, giving counting errors of less than 2% for either ^{3}H- or ^{14}C-, were done in all cases.

[2] DHA, dehydroepiandrosterone; Δ^{4}, Δ^{4}-androstenedione; T, testosterone; Δ^{5}, Δ^{5}-pregnenolone; P, progesterone; 17α, 17α-hydroxyprogesterone.

Table 5 (Cont.)

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[3] Prior to derivatization 5 mg of the putative steroids were added. Acetates of DHA, T, Δ^5 and 17α were made by reaction with 2:1-pyridine:acetic anhydride; for Δ^4 and P methyloximes were formed by reaction with a 2% solution of methoxylamine HCl in pyridine.

support the presence of the putative steroids within the captured effluents. It should also be noted that these data were generated by a single set of recrystallizations, each sampled only once for each step of purification; the variability of the ratios between crystallizations would likely have been smaller if duplicates or triplicates had been run. The results support the identity of the captured radioactivity with the peak assignments given in Figure 12 with the possible partial exceptions of dehydroepiandrosterone and progesterone. (Other data, reported in Chapter 6 and the Appendix tend to mollify even these exceptions.)

Counting and GC data for the metabolism of Δ^5-pregnenolone were combined and analyzed by the use of the procedures discussed in Chapter 6 and the computer program detailed in the Appendix.

CHAPTER 3

EXPERIMENTS ON THE EFFECTS OF DIRECT INJECTION OF o,p'-DDT INTO NEONATAL MALE RATS

The approach summarized in Figure 4 implied several possible divisions of experimental results: by route of administration, by castration state or by measured variable. The most coherent discussion of the findings seemed to be based on the route of administration. Therefore, the next two chapters will describe the results of the physiological measurements made on animals dosed by direct (Chapter 3) and indirect (Chapter 4) means. A coordinating discussion including conclusions and postulations for future experimental directions comprises Chapter 5. Due to the different nature and development of the studies done on testicular incubations they will be discussed separately in Chapter 6.

I. Organ Weights in Intact Male Rats and in Neonatally Castrated Adult Male Rats Neonatally Injected with o,p'-DDT:

The initial experiment in these studies was an attempt to determine the existence in male rats of a hypothalamic imprinting by o,p'-DDT which was similar to that which had been reported in females (53). It was decided, on the basis of observations made on animals imprinted with steroids (57-61,63-67), that alterations of growth and/or organ weight should be demonstrable if steroidogenic derangement occurred. This would be true if the derangement was caused by either a direct action on the steroidogenic endocrine glands or by an indirect action via the hypothalamus or the liver. Neonatally castrated animals were also included in the experiment to investigate the possibility that endogenous testosterone was involved in either the production or the amelioration of any observable effect of neonatally administered o,p'-DDT. Animals treated with 17β-estradiol-17-valerinate (EV) were also included. They served as positive controls, confirming the sensitivity of the animals used to imprinting and providing a comparison of the extremes seen in normal and imprinted animals. Estradiol was chosen as a control over testosterone because previous reports supported the idea that o,p'-DDT acted as a weak estrogen (22,24,26,27,146-148,150,162).

Within 6 hours after delivery pups were placed into litters of 8 pups each and about 1/3 of the males were castrated. The castrated and intact litters were then placed into 3 treatment groups and injected on each of days 1-5 of age. Controls received injections of 0.05 ml sesame oil; DDT-treated animals received injections of 0.05 ml of sesame oil containing 400 ug o,p'-DDT; EV treated animals received injections of 0.05 ml sesame oil containing 40 ug 17β-estradiol-17-valerinate. Weights were determined at regular intervals throughout development. The rats were sacrificed between 81 and 110 days of age at which time the body and organ weights were recorded along with any obvious morphological abnormalities.

Body weights measured during development did not differ noticeably among the groups until after 35-40 days of age. At this time the growth of estrogen-treated animals began to lag behind the other groups; castrates lagged behind intact animals in all groups after 45-55 days of age. In neither the intact groups nor the castrated groups did neonatal treatment with o,p'-DDT alter the course of weight gain. The effect of estrogen treatment in both intact and castrate groups was marked, in accord with the observations of Oštádalová and Pařízek (197).

The final body weights and organ weights, normalized by body weight, of all the groups are shown in Table 6. Body weights for intact control and intact DDT-treated animals were statistically indistinguishable. Neonatally castrated control and DDT-treated groups were likewise similar to each other though the influence of castration was marked. In both intact and castrated states EV caused a very significant ($p < 0.0005$) decrease in body weight, roughly 35% in both cases.

The glands directly involved in the production of trophic and steroid hormones, the pituitary, adrenals and testes, demonstrated no differences between the control and DDT-treated groups in either the intact or castrated states. Estrogen on the other hand, acted on the adrenals

Table 6

Effects of Neonatal Injection with o,p'-DDT or Estradiol[1] on Body[2] and Organ Weights of Intact and Neonatally Castrated Male Rats

Percent of Body Weight[3]	Intact			Castrated		
	Control(11) (422±22)	DDT(5) (404±20)	EV(11) (298±20)‡	Control(5) (240±23)	DDT(7) (249±19)	EV(6) (222±11)‡
Pituitary x 10^3	2.75±0.20	2.59±0.11	2.81±0.18	3.76±0.18	4.12±0.70	3.42±0.46
Adrenals x 10^2	1.27±0.10	1.23±0.09	1.99±0.30‡	1.83±0.29	1.95±0.15	2.46±0.24#
Testes	0.84±0.07	0.87±0.06	0.25±0.06‡	---	---	---
Seminal } x 1	0.28±0.02	0.30±0.03*	0.04±0.02‡	---	---	---
Vesicles } x 10^3	---	---	---	6.71±2.14	4.84±1.08	32.9±5.7‡
Ventral } x 1	0.11±0.02	0.12±0.01	0.02±0.02‡	---	---	---
Prostate } x 10^3	---	---	---	3.10±0.71	2.57±0.59	3.06±0.17
Liver	3.71±0.30	3.74±0.32	3.39±0.21 t	3.35±0.25	3.25±0.27	3.92±0.10#
Kidneys	0.76±0.05	0.73±0.05	0.66±0.05‡	0.62±0.06	0.60±0.03	0.68±0.03*

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[1] Injected s.c. with 0.05 ml sesame oil, 400 ug o,p'-DDT in 0.05 ml sesame oil or 40 ug estradiol valerinate in 0.05 ml sesame oil on days 1-5 of age. Reared in litters of 8 pups each and sacrificed at 81 to 110 days of age.

Table 6 (Cont.)

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[2] Body weights given in grams in parentheses under each heading.

[3] All data are given as mean weights or mean % of body weight ± 1 standard deviation; numbers in parentheses after the treatment designation are the numbers of animals examined; comparisons are all versus the appropriate control groups and are made by unpaired Student's t tests, * $p < 0.05$, t $p < 0.01$, # $p < 0.005$, ‡ $p < 0.0005$.

of both intact and castrated animals to cause elevations of weights to above control group levels. While neonatal castration itself caused marked hypertrophy to above intact weights of both the adrenal and pituitary in both control and DDT-treated groups, the effects of neonatal castration and EV were nearly additive. This could be seen by comparing the intact EV-treated group with both the castrated control group and the castrated EV-treated group. Estrogen also markedly diminished testicular weight suppressing it roughly 70% below control values.

The accessory sex tissues, the seminal vesicles and ventral prostate, should reflect the output of hormonally active gonadal androgens (198). DDT only slightly increased ($p < 0.05$) the weight of the seminal vesicles in intact animals in comparison to controls; it did not effect the seminal vesicles of castrated rats when compared to similarly castrated controls. Prostate weight in both intact and castrated animals was unaffected by DDT administration. Castration alone produced a marked decrease in sex-accessory weights in both DDT and control groups. In the estrogen treated groups, however, the consequences of EV treatment and/or castration were more complex. First, estrogen suppressed intact prostate weights into the range seen in control castrates; superposition of castration and EV did not effect prostate weights more than castration alone. Second, estrogen decreased ($p < 0.0005$) the seminal vesicle weights of intact animals into the range seen in EV-treated neonatally castrated rats. Third, the seminal vesicular weights of neonatally castrated animals was increased by EV treatment 5-6 fold ($p < 0.0005$) over the weights observed for castrated controls. These seminal vesicles (from EV-treated neonatal castrates) are, however, morphologically abnormal, being more muscular than those in the other castrates and retaining an integral association with what are probably the vestiges of parts of the Müllerian ductal system. These tissues in the other castrated groups appear merely immature in comparison to intact controls.

Liver and kidney weights are also affected by steroidal status (199) and are, in addition, sensitive to potential hepatic inducers (5). They were unaffected by the administration of o,p'-DDT to either intact or castrated animals. Again castration removed what is an anabolic influence since the liver and kidneys of castrates were slightly lighter than those of the intact animals. Neonatal estrogen again exerted paradoxic effects by diminishing the organ weights in intact animals into the control castrate range while increasing the organ weights in castrated animals toward, or even in excess of, intact control levels.

Changes due to estrogen treatment were marked, as anticipated. Inconsistencies of the changes in intact animals with those seen with castration alone should not, however, allow the misinterpretation that estrogen treatment of intact males is nothing more than chemical castration (200); body, pituitary and seminal vesicle weights in intact EV-treated animals do not concur with those found in control castrates. The issue is confused by the changes seen in EV-treated neonatal castrates where body, adrenal, seminal vesicular, liver and kidney weights are increased rather than suppressed. The continued presence of injected estrogen at levels sufficient to suppress body weight and to stimulate adrenal weight in both intact and castrated animals, to suppress gonadal function in intact animals and to induce preservation of Müllerian structures serves as an obvious alternative to imprinting as an explanation for the results observed.

Clearly, the changes due to neonatal treatment with a total of 2 mg of o,p'-DDT were minimal. Several interpretations were therefore possible: no effects occurred; effects were too subtle to be detected by such gross methodology; or, effects occurred earlier in development and were not measurable at the time of this experiment. The first interpretation was presumptuous at this point and finalistic, in addition; it could lead to no further experimentation. The second interpretation dictated the use of more sensitive measurements in subsequent experiments. The third interpretation implied the need for the examination of some sort of developmental time course.

These last two suggestions formed the basis for the next experiment.

II. Developmental Time Course and Dose-Response to Neonatally Administered o,p'-DDT:

In addition to probing body and organ weights as a function of development, this experiment included examination of organ histology and measurements of serum corticosterone, to monitor adrenal output, and serum LH, to measure pituitary function. It also included several levels of o,p'-DDT exposure. The inclusion of more than one dose level was meant to generate dose-response data on any effects encountered. It was also meant to minimize the potential effect of multiple antagonistic responses on the conclusions drawn from the experimental results, e.g., to prevent hepatic induction from completely eliminating a form of DDT which imprints the hypothalamus. Positive control groups were injected with either EV or testosterone-17-propionate (TP). Estrogen was included for the reasons cited previously while testosterone was included to provide an apparently less drastic though similar form of imprinting (59) which has been postulated to be mediated by the estrogen formed by localized hypothalamic aromatization of testosterone (87). Due to the size of the proposed experiment neonatal castrates were not included.

Following parturition, the intact neonatal males were placed into litters of 8 pups each. Thirty-six litters were divided into 9 groups, 6 vehicle-treated control (C) litters, 6 EV-treated litters, 6 TP-treated litters and 18 litters treated with o,p'-DDT - 3 litters at each of 6 dose levels. The chemicals were administered s.c. in 5 equal daily doses, each contained in 0.05 ml of sesame oil, given from the day of birth until 4 days after birth. Dosages were as follows:

Group	Chemical	Daily Dose (ug)	Total Dose (ug)
C	None	0	0
I	o,p'-DDT	400	2000
II	o,p'-DDT	200	1000
III	o,p'-DDT	100	500
IV	o,p'-DDT	20	100
V	o,p'-DDT	10	50
VI	o,p'-DDT	2	10
EV	Estradiol	40	200
TP	Testosterone	200	1000

After weaning, animals were caged 6 to a cage. Body weights were recorded throughout development. On days 25, 50, 75 and 110 five to six animals from each DDT-treatment level and 8-11 animals from each of the control, EV and TP groups were decapitated between 12.00 and 16.00 (EST).[1,2]

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[1] Since the experiment was actually conducted in two halves started 5 days apart, only 5-6 animals from each of 3 DDT-treated groups and 5-6 animals from each of the control, EV and TP groups were processed on any one day.

[2] On day 110 groups C and I and ½ of groups EV and TP were

Animals from all the groups were alternately sacrificed in order to minimize any time bias between groups. Tissues were rinsed in Buffer I (see Materials and Methods IV.F.1.), blotted, weighed, divided and placed into 10% neutral formalin.

Data for the halves (see footnote 1, p. 110) of each group of controls, EV-treated or TP-treated animals were checked for uniformity by applying Student's t-test to the two halves. After ascertaining uniformity, the values from the half-groups were combined. All organ weights were normalized against total live body weight before means or errors were calculated. Mean values for each individual group of treated animals were then tested by Student's t-tests against the means of the combined control group, C.

A. Body and Organ Weights:

Body weight gain in all groups prior to 32-35 days of age was uniform. By about day 40 estrogen treated animals began to lag behind in growth and by day 110 were fully 30% lighter than control rats. Testosterone-treated animals lagged slightly in growth between days 40 and 85 but were nearly normal by day 110. Body weight gain of DDT-treated rats was intermediate between that of the controls and that of the TP group from days 40 to 80. Beyond 80 days, groups I-VI showed a somewhat slower growth with groups

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sacrificed at 10.00 while the remaining animals were killed at 19.00 (EST).

II, III and V demonstrating significantly lower body weights by day 110.[3] No relationship between DDT dosage and growth, as measured by body weight was evident. Body weights for days 25, 50, 75 and 110 are summarized in the graph in Figure 13.

The mean normalized organ weights of the animals described in Figure 13 are shown in Figures 14-20.

Normalized pituitary weights (Figure 14) decreased uniformly with time. Estrogen treatment caused a significant rise, above control levels, in pituitary weight which was only evident before day 110 of age. Testosterone did not alter pituitary weight gain. Although several isolated changes were seen in the DDT-treated animals those on day 110 may be explained by the lower body weight of the animals (the mean raw weights of the pituitaries were not different from controls). The significant value for group II on day 25 was probably by chance due to the size of the experiment. No consistent pattern of fluctuation of means about the control mean was evident for the DDT-treated animals.

Normalized testicular weights for pairs of testes (Figure 15) in both control and DDT-treated groups demonstrated the abrupt hypertrophy associated with puberty,

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[3] Group III contracted respiratory infection shortly before the termination of the experiment; most of the actual weight loss seen on day 110 in that group and many of the deviations in normalized organ weights are undoubtedly due to illness.

Figure 13. Body Weights of Neonatally Injected Male Rats

Intact male rats were injected daily, s.c., on days 1-5 of age with 0.05 ml of sesame oil which contained 1/5 of the following total dosages: C = vehicle only, control; I = 2000 ug o,p'-DDT; II = 1000 ug o,p'-DDT; III = 500 ug o,p'-DDT; IV = 100 ug o,p'-DDT; V = 50 ug o,p'-DDT; VI = 10 ug o,p'-DDT; EV = 200 ug 17β -estradiol-17-valerinate; TP = 1000 ug testosterone-17-propionate. The animals were raised in litters of 8 pups and had free access to water and food at all times. Between 12.00 and 16.00 on the days specified the animals were weighed, decapitated and dissected; fresh organ weights were then determined. (Note: on day 110 groups C and I and ½ of groups EV and TP were sacrificed at 10.00 while the remaining animals were killed at 19.00 (EST). This does not alter the interpretation of the results or the contrasts made except for the liver which shows marked diurnal weight fluctuations.)

The data shown are means +1 standard deviation; groups I-VI contained 5-6 rats each while groups C, EV and TP each contained 8-11 animals. Tests of statistical difference from the mean of the control group, C, on the given days were made by unpaired Student's t-tests. Probabilities of the differences seen occurring by chance are noted for those probabilities of less than 5%: a, $p < 0.05$; b, $p < 0.025$; c, $p < 0.01$; d, $p < 0.005$; e, $p < 0.0005$.

FIGURE 13

BODY WEIGHTS OF NEONATALLY INJECTED MALE RATS

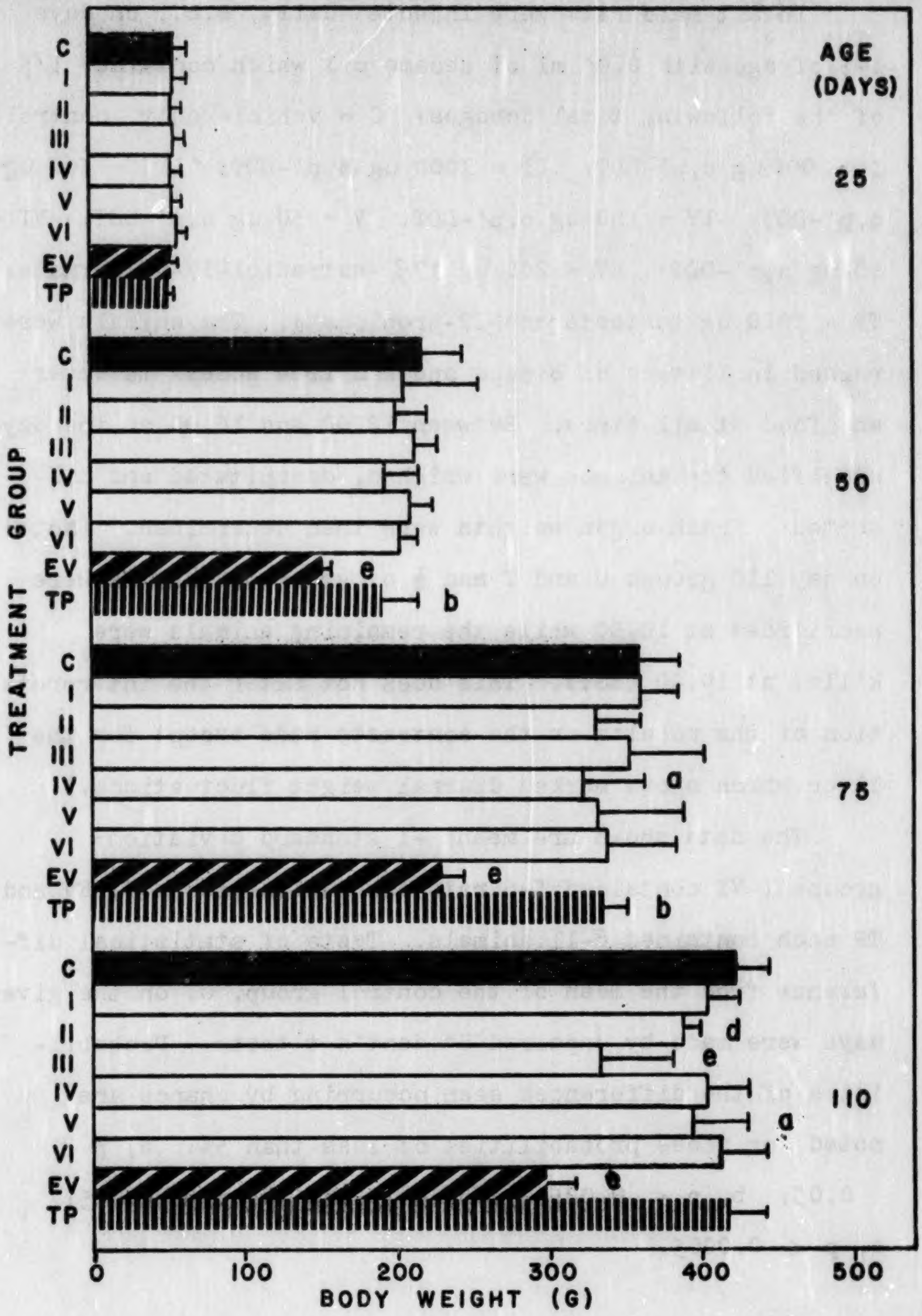


Figure 14. Organ Growth in Neonatally Injected Male Rats: Pituitary

The pituitary weights, expressed as 1000 x percent of body weight which are displayed are those of the animals listed in Figure 13. Organ weights were normalized by body weight for each individual animal prior to calculating the group means which are shown (+1 standard deviation). Group sizes were 3-6 for groups I-VI and 8-11 for groups C, EV and TP. Group means were tested individually by Student's t-test for differences from the control group, C, mean on each day listed. Statistical probabilities of less than 0.05 are noted: a, $p < 0.05$; b, $p < 0.025$; c, $p < 0.01$; d, $p < 0.005$; e, $p < 0.0005$.

FIGURE 14

ORGAN GROWTH IN NEONATALLY INJECTED MALE RATS

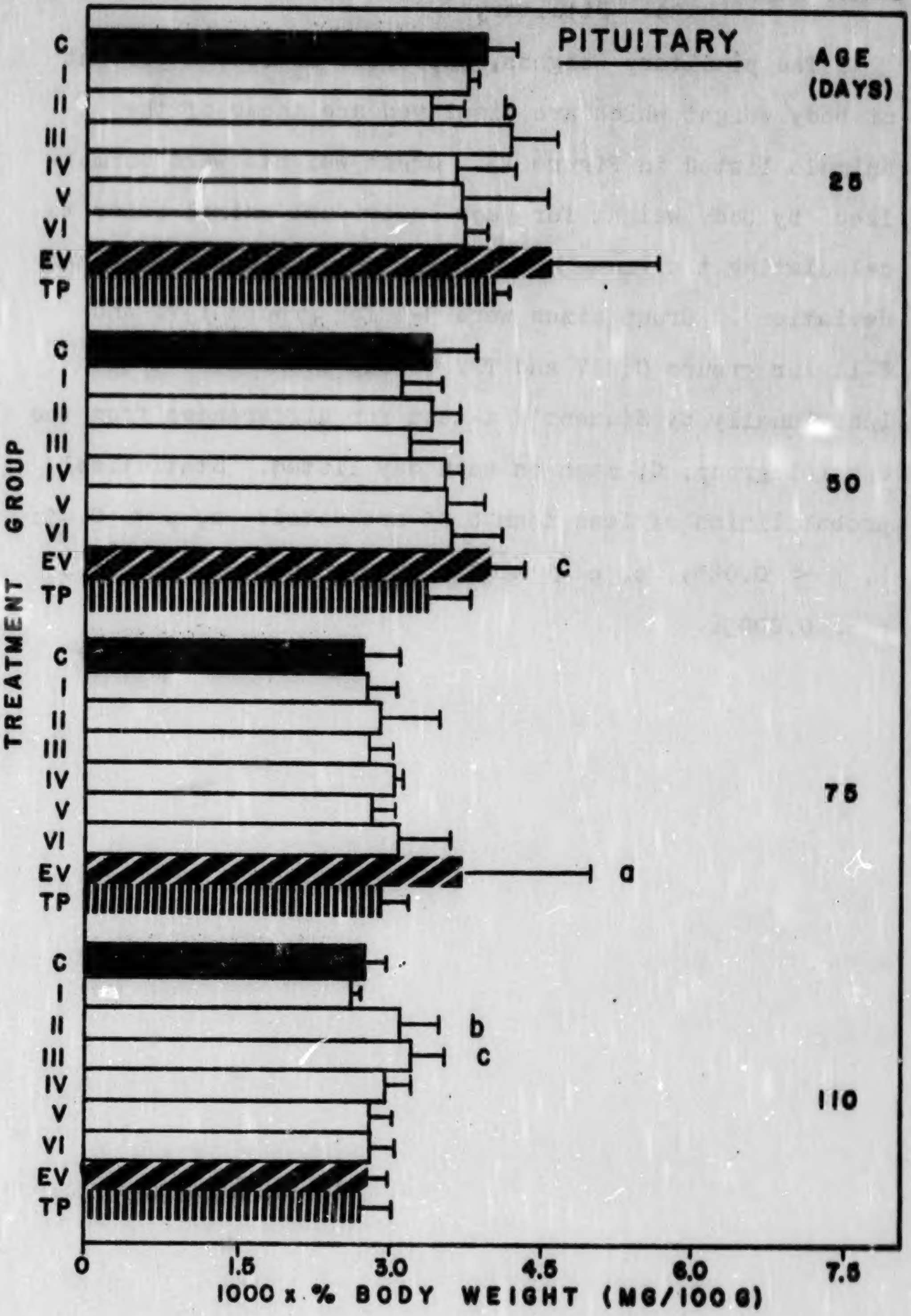
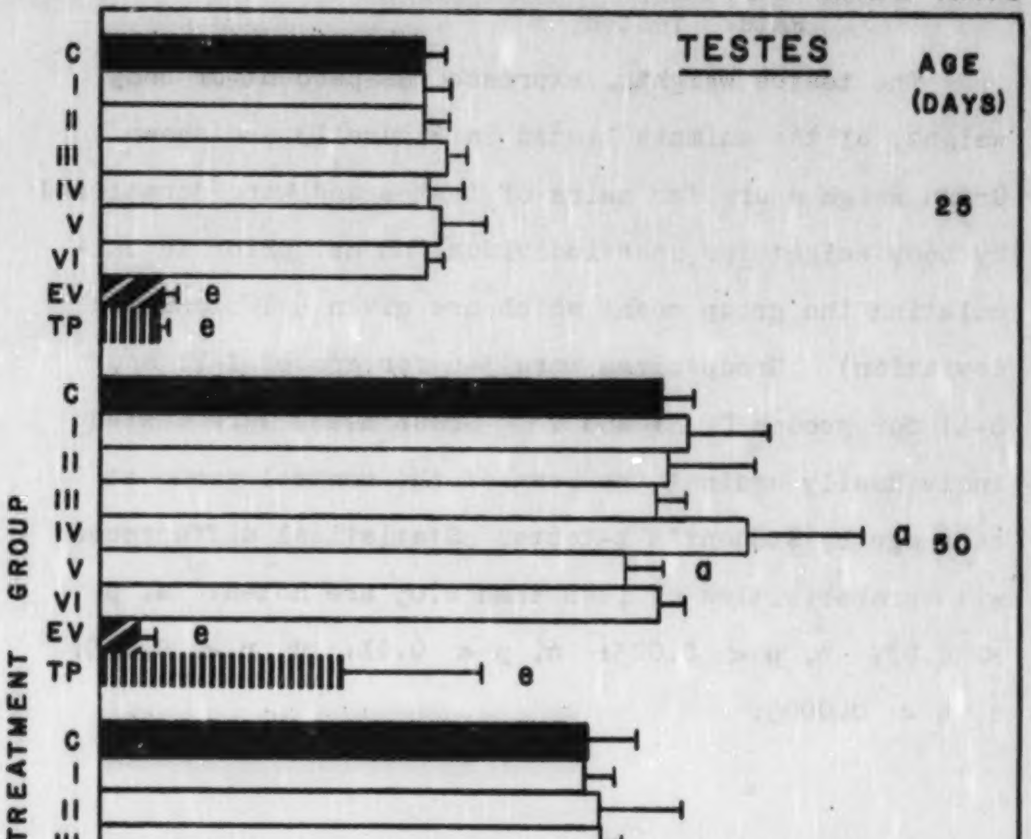


Figure 15. Organ Growth in Neonatally Injected Male Rats: Testes

The testes weights, expressed as percent of body weight, of the animals listed in Figure 13 are shown. Organ weights are for pairs of testes and were normalized by body weight for each individual animal prior to calculating the group means which are given (+1 standard deviation). Group sizes were 5-6 for groups I-VI and 8-11 for groups C, EV and TP. Group means were tested individually against the mean of the control group at each age by Student's t-tests. Statistical differences with probabilities of less than 0.05 are noted: a, $p < 0.05$; b, $p < 0.025$; c, $p < 0.01$; d, $p < 0.005$; e, $p < 0.0005$.

FIGURE 15

ORGAN GROWTH IN NEONATALLY INJECTED MALE RATS



activation of steroidogenesis and active spermatogenesis, between 25 and 50 days of age; thereafter body weight rose proportionately faster than testicular weight and the normalized organ weights slowly decreased. As in the case of the pituitary, several isolated significant deviations from the control mean were seen in the DDT-treated groups; all could be explained by differences in body weights. This was not true, however, for treatment with EV or TP. Estrogen markedly suppressed testicular weight throughout the period studied; only a slight alleviation of the effect was seen between days 50 and 75. Testosterone yielded marked suppression throughout the experiment. Still, some sort of recovery, which coincided with the increased body weight seen after 75 days of age, apparently took place since normalized testicular weights for this group slowly rose beyond 50 days of age. This apparent recovery in TP-treated rats is in concert with similar observations in mice (59).

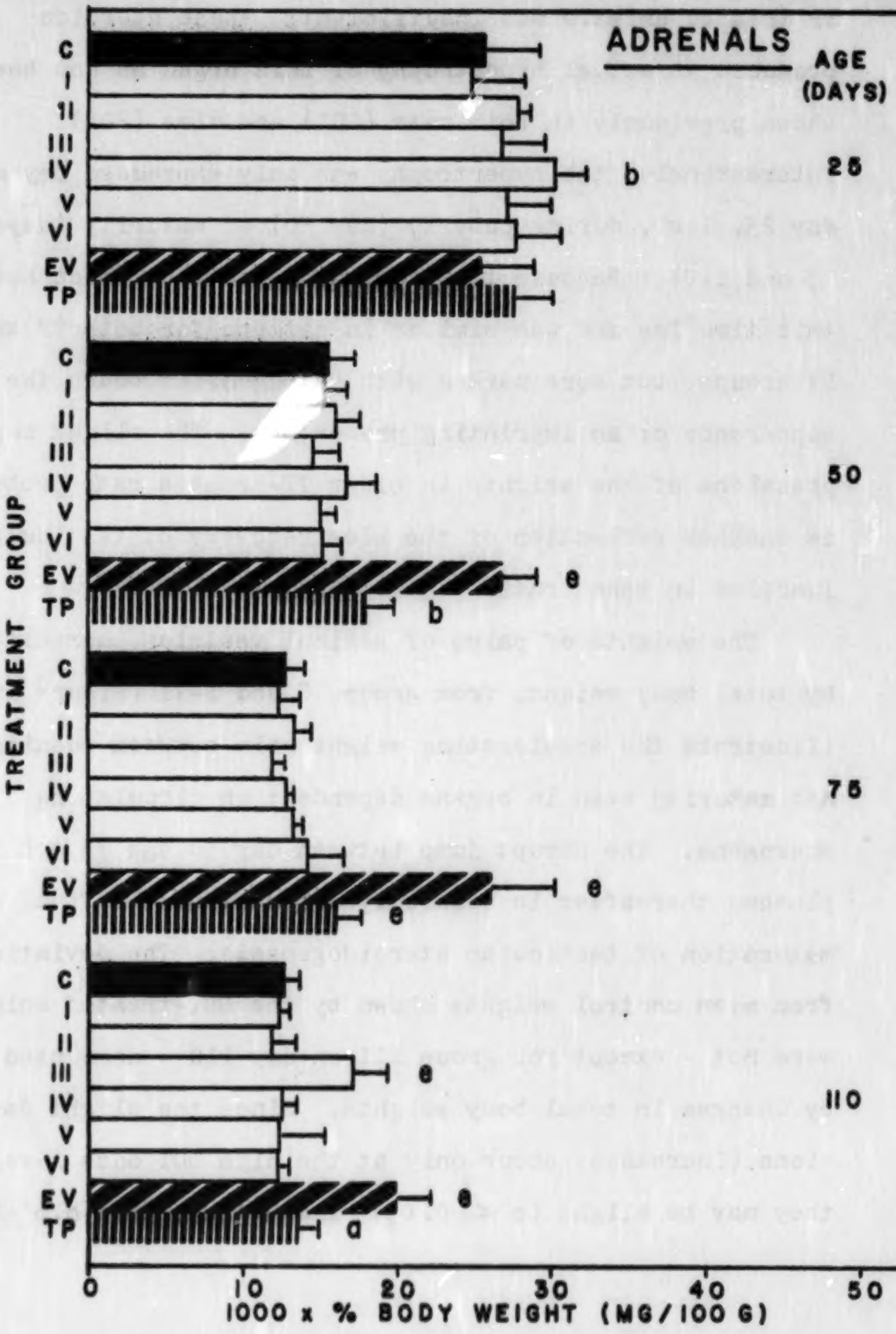
Normalized adrenal weights for pairs of adrenals (Figure 16) declined abruptly for all but the estrogenized animals between days 25 and 50; they decreased in proportion to total weight only slowly beyond day 50. Again DDT appeared ineffective in altering weights although group IV on day 25 did show an increase above control levels which was not explicable on the grounds of differences in total body weight. The majority of the difference seen with group III on day 110 was, however,

Figure 16. Organ Growth in Neonatally Injected Male Rats: Adrenals

The adrenal weights, expressed as 1000 x percent of body weight, of the animals listed in Figure 13 are shown. Organ weights are for pairs of glands and were normalized by body weight for each individual animal prior to calculating the group means given (+1 standard deviation). Group sizes were 5-6 for groups I-VI and 8-11 for groups C, EV and TP. Group means were tested individually against the mean of the control group at each age by Student's t-tests. Statistical differences with probabilities of less than 0.05 are noted: a, $p < 0.05$; b, $p < 0.025$; c, $p < 0.01$; d, $p < 0.005$; e, $p < 0.0005$.

FIGURE 16

ORGAN GROWTH IN NEONATALLY INJECTED MALE RATS



accounted for by a decreased body weight (see footnote 3, p. 112). The same explanation for the results in EV and TP treated animals was insufficient; these steroids produced an actual hypertrophy of this organ as has been shown previously in both rats (201) and mice (202). Interestingly, the hypertrophy was only expressed beyond day 25, i.e., during puberty (day 50) or maturity (days 75 and 110). Because the expression of this effect has this time lag and was similar in pattern for both TP and EV groups, but more marked with estrogen, it bears the appearance of an imprinting phenomenon. The slight suppressions of the weights in older TP-treated rats probably is another reflection of the slow recovery of testicular function in these rats.

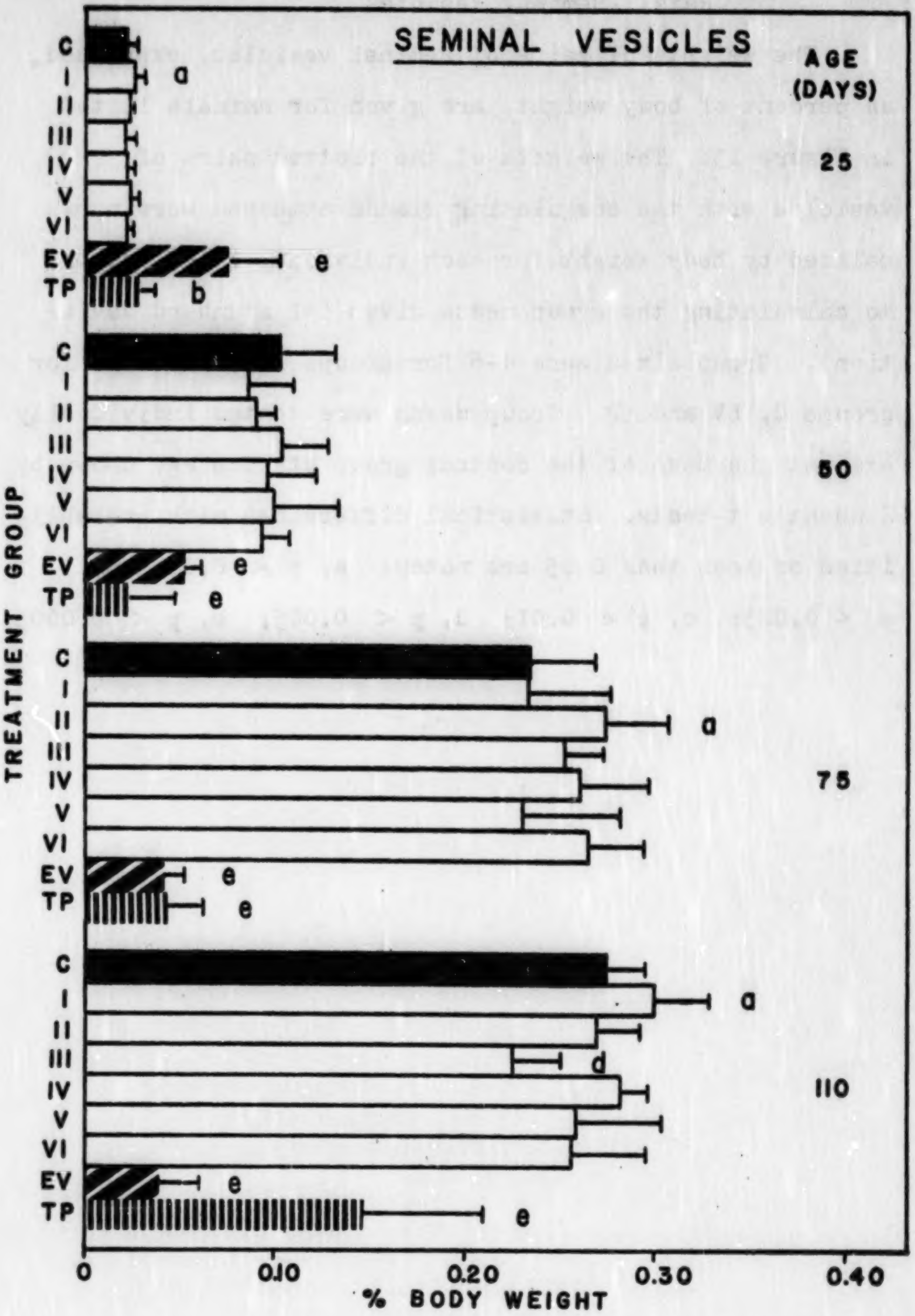
The weights of pairs of seminal vesicles, normalized by total body weight, from groups C and I-VI (Figure 17) illustrate the accelerating weight gain between weaning and maturity seen in organs dependent on circulating androgens. The abrupt jump between day 50 and 75 and the plateau thereafter is assuredly a result of the final full maturation of testicular steroidogenesis. The deviations from mean control weights shown by the DDT-treated animals were not - except for group III on day 110 - accounted for by changes in total body weights. Since the slight deviations (increases) occur only at the high DDT dose levels they may be slight ($p < 0.05$) indications that o,p'-DDT

Figure 17. Organ Growth in Neonatally Injected Male Rats: Seminal Vesicles

The weights of pairs of seminal vesicles, expressed as percent of body weight, are given for animals listed in Figure 13. The weights of the blotted pairs of vesicles with the coagulating glands attached were normalized by body weight for each individual animal prior to calculating the group means given (+1 standard deviation). Group sizes were 4-6 for groups I-VI and 8-11 for groups C, EV and TP. Group means were tested individually against the mean of the control group at each age shown by Student's t-tests. Statistical differences with probabilities of less than 0.05 are noted: a, $p < 0.05$; b, $p < 0.025$; c, $p < 0.01$; d, $p < 0.005$; e, $p < 0.0005$.

FIGURE 17

ORGAN GROWTH IN NEONATALLY INJECTED MALE RATS



is a weak imprinting agent which, paradoxically, causes increased adult gonadal output.

The effect of estrogen was marked ($p < 0.0005$ on all days examined). On day 25 an hypertrophy similar to that seen in estrogen-treated neonatal castrates (experiment 1) and reported by Freud (203) was observed - again, abnormal morphology may hold the explanation for this result. The raw weight of the vesicles in these animals continue to increase slowly until sometime between 50 and 75 days of age after which it stabilizes; in no case, however, does it approach normal levels, indicating a lack of, or insensitivity to, circulating androgens.

Testosterone also exerts a marked effect on this tissue beyond 25 days of age (day 25 $p < 0.025$, days 50-110 $p < 0.005$). But, as in the cases of the testes and adrenals, it appears to be mainly a retardation of development, maturation being completed sometime after 110 days of age in these animals. The results were suggestive of residual titers of injected TP which only fall below values which suppress LH levels after 75-110 days of age. If these TP titers were, in addition, insufficient to support gonadal function (normal function would antagonize rises in adrenal weight (4,204)) they could explain the results discussed thus far for testosterone treated animals.

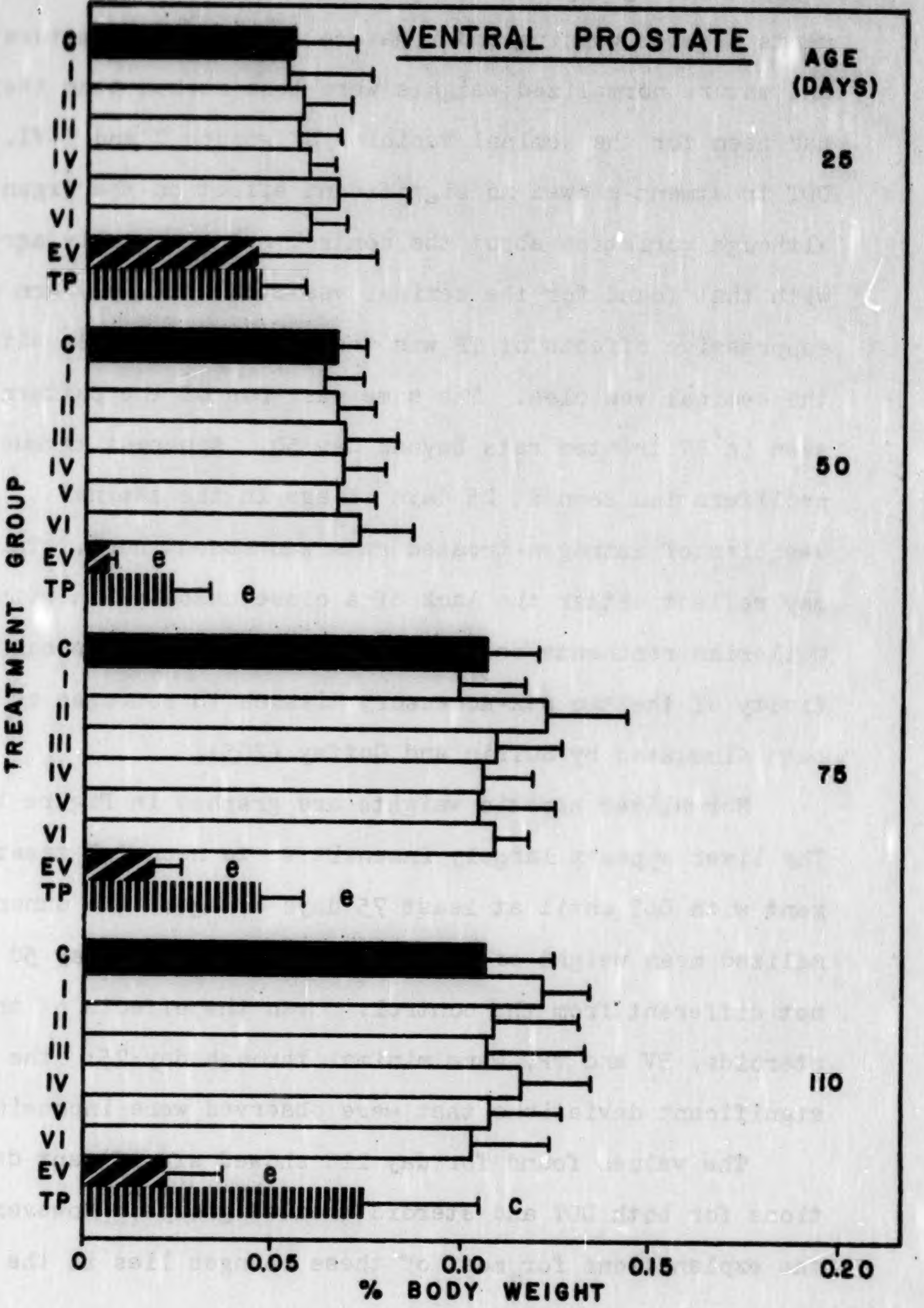
The ventral prostate, normalized weights of which are shown in Figure 18, would be expected to demonstrate the

Figure 18: Organ Growth in Neonatally Injected Male Rats: Ventral Prostate

The weight of the ventral portion of the prostate gland, expressed as percent of body weight, is shown for the animals described in Figure 13. The weight of the tissue was normalized by body weight for each individual animal prior to calculating the group means (+1 standard deviation) depicted. Group sizes were 4-6 for groups I-VI and 8-11 for groups C, EV and TP. Group means were tested individually against the mean of the control group at each age shown by Student's t-tests. Statistical differences with probabilities of less than 0.05 are noted: a, $p < 0.05$; b, $p < 0.025$; c, $p < 0.01$; d, $p < 0.005$; e, $p < 0.0005$.

FIGURE 18

ORGAN GROWTH IN NEONATALLY INJECTED MALE RATS



same patterns as the seminal vesicles. This was generally true for all groups but that treated with EV. The increments between weaning and immature and between immature and mature normalized weights were less marked than they had been for the seminal vesicles of groups C and I-VI. DDT treatment showed no significant effect on the organ although variation about the control mean generally agreed with that found for the seminal vesicles. The pattern of suppressive effects of TP was the same as that seen with the seminal vesicles. The same was true of the pattern seen in EV treated rats beyond day 50. Apparent tissue proliferation seen at 25 days of age in the seminal vesicles of estrogen-treated rats was absent here. This may reflect either the lack of a close association with Müllerian remanents or an innate difference in the sensitivity of the two sex-accessory tissues to estrogen as has been discussed by Sufrin and Coffey (205).

Normalized hepatic weights are graphed in Figure 19. The liver appears largely insensitive to neonatal treatment with DDT until at least 75 days of age; the unnormalized mean weight of the liver in group IV on day 50 was not different from the control. Even the effects of the steroids, EV and TP, were minimal through day 75; the significant deviations that were observed were inconsistent.

The values found for day 110 showed significant deviations for both DDT and steroid treated groups. However, the explanations for most of these changes lies in the

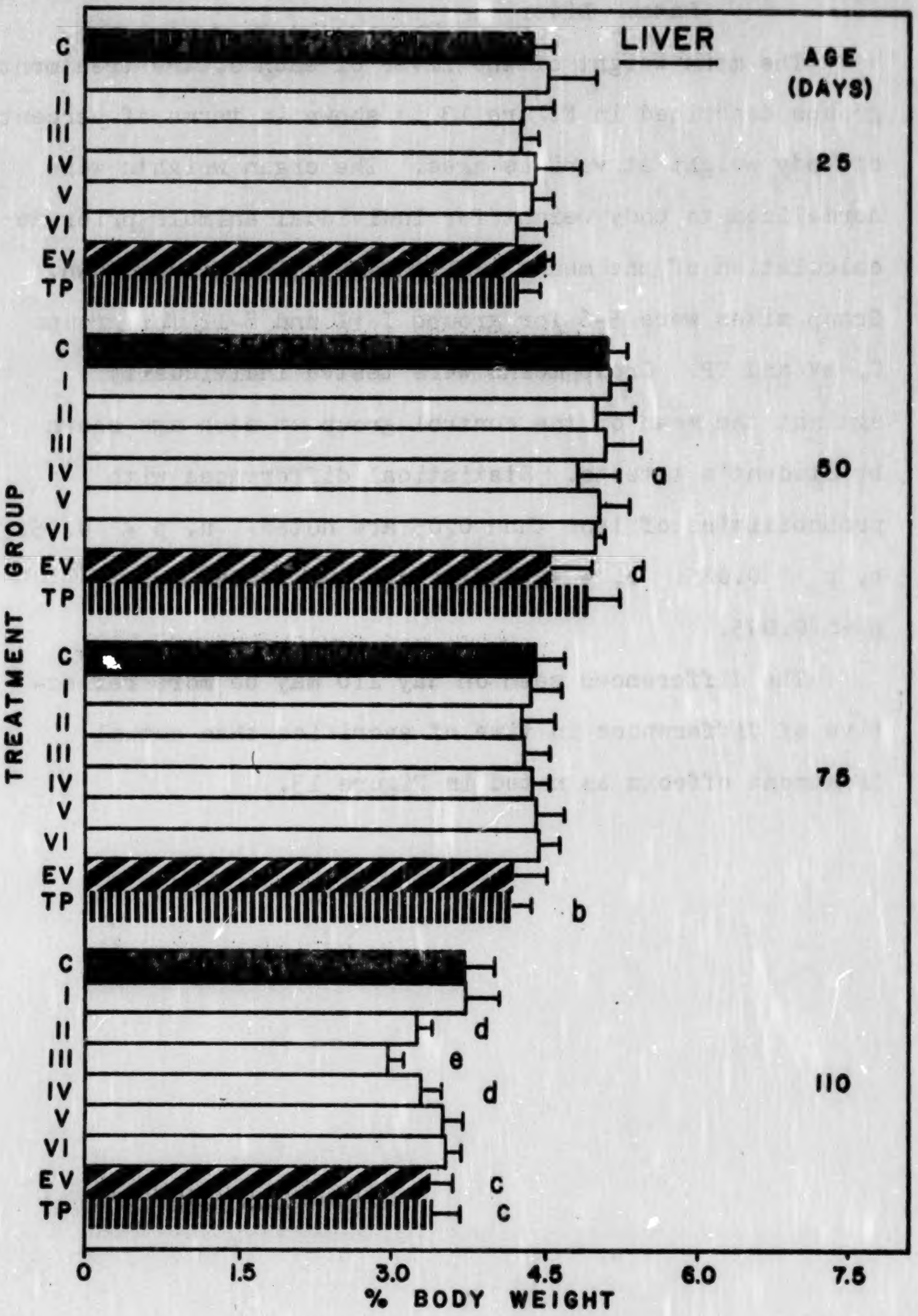
Figure 19. Organ Growth in Neonatally Injected Male Rats: Liver

The mean weight of the liver of each of the treatment groups described in Figure 13 is shown in terms of percent of body weight at various ages. The organ weights were normalized to body weight for individual animals prior to calculation of the means (+1 standard deviation) shown. Group sizes were 5-6 for groups I-VI and 8-11 for groups C, EV and TP. Group means were tested individually against the mean of the control group at each age shown by Student's t-tests. Statistical differences with probabilities of less than 0.05 are noted: a, $p < 0.05$; b, $p < 0.025$; c, $p < 0.01$; d, $p < 0.005$; e, $p < 0.005$.

The differences seen on day 110 may be more reflective of differences in time of sacrifice than actual treatment effects as noted in Figure 13.

FIGURE 19

ORGAN GROWTH IN NEONATALLY INJECTED MALE RATS



altered experimental procedure followed on that day and in the normal diurnal weight fluctuations demonstrated by the liver (206). In order to allow incubations of the testes to be done on these rats sacrifice times were altered from the normal time of 12.00-16.00 (EST) used on previous days (see footnote 2, p. 110). The data from the halves of groups EV and TP were compared but failed to yield significant t values; therefore, the data were combined. The decreases of the normalized organ weights to below the control mean in groups II-IV, EV and TP probably best reflected the 4-9 hour difference in sampling times rather than actual effects of DDT or steroid exposures. The magnitude and time course of the change agree completely with those observed by Potter et al. (206) in studies of feeding and corticosterone secretion.

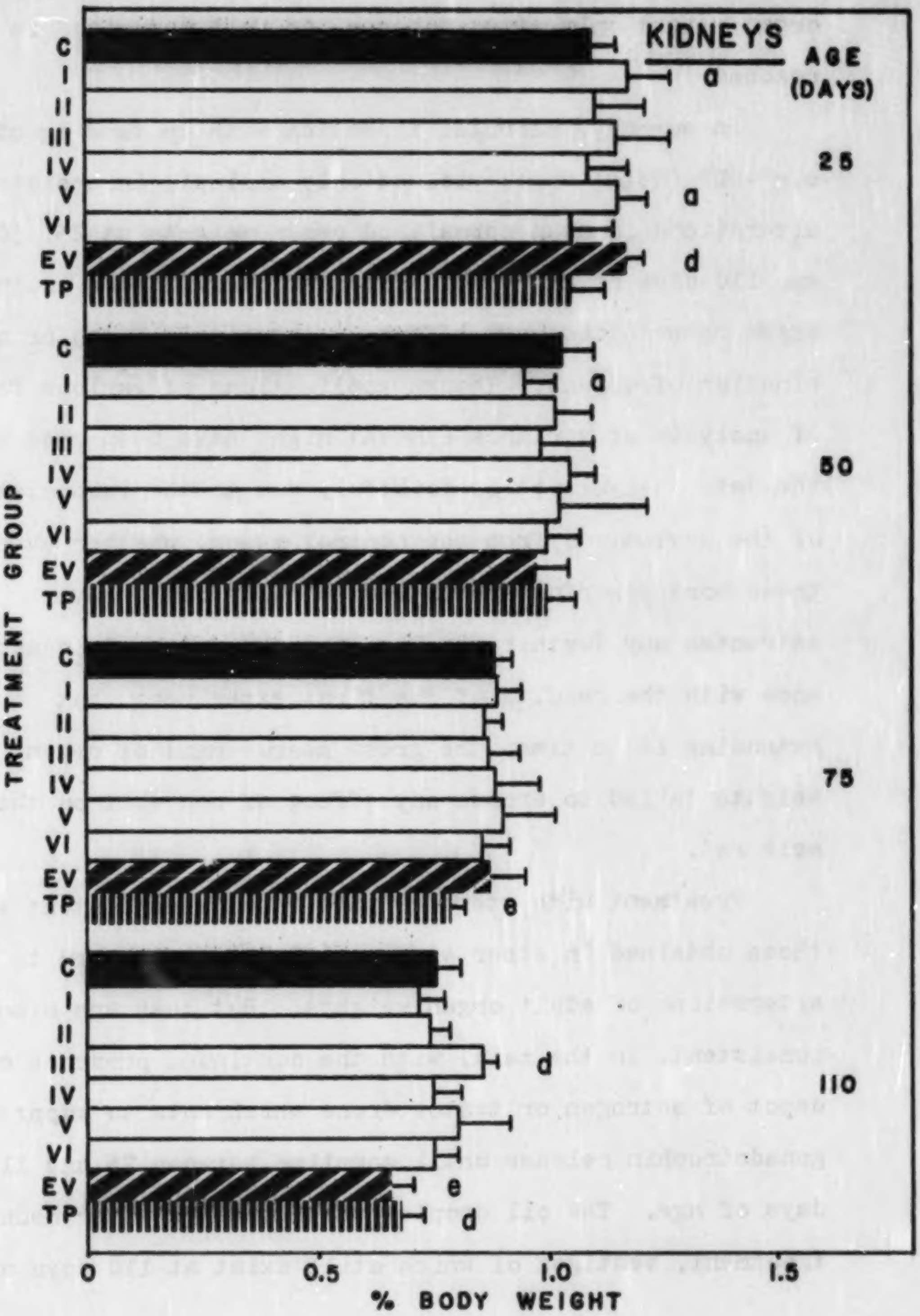
Finally, weights for pairs of kidneys, normalized to body weight, are shown in Figure 20. A consistent decrease with age in the normalized organ weight was demonstrable in all treatment groups; the value for group III on day 110 was explained by decreased body weight. In fact, the greatest portion of each of the deviations from the group C mean before day 75 are explicable by differences in body weights since data on raw organ weights indicate minimal deviations from that of group C. Treatment with TP did depress kidney weights after day 50 but the effect of estrogen treatment was significant for both raw and normalized weights only on day 110. Again, as has been the

Figure 20. Organ Growth in Neonatally Injected Male Rats: Kidneys

The mean weights of pairs of kidneys, expressed as percent of body weight, are shown for the treatment groups described in Figure 13 for various ages. Pairs of organs were blotted and weighed, the weights being normalized by body weight for each individual animal prior to calculation of the group means (+1 standard deviation) given. Group sizes were 5-6 for groups I-VI and 8-11 for groups C, EV and TP. The individual group means for each age were tested against the control group means by Student's t-tests; statistical differences with probabilities of less than 0.05 are noted: a, $p < 0.05$; b, $p < 0.025$; c, $p < 0.01$; d, $p < 0.005$; e, $p < 0.0005$.

FIGURE 20

ORGAN GROWTH IN NEONATALLY INJECTED MALE RATS



case for most of the organs examined, the actions of the steroids become manifest as alterations in mean normalized organ weight only after puberty, or full maturity, is reached.

In summary, neonatal injection with up to 2 mg of o,p'-DDT (total dose) yielded only minimal, inconsistent alterations in mean normalized organ weights at 25, 50, 75 and 110 days of age. No consistent correlations could be drawn between dose and effect on any single organ or combination of organs. Though applications of various forms of analysis of variance (ANOVA) might have been used in the data analysis it is doubtful, due to the inconsistency of the deviations from the control means, whether even these more powerful statistical methods could have extracted any further results from the data. In concordance with the results of the first experiment, but extending it in time, the gross measurement of organ weights failed to expose any effect of o,p'-DDT on the male rat.

Treatment with steroids gave results consistent with those obtained in other studies (59,207) in regard to alterations of adult organ weights. But they are also consistent, in the main, with the continued presence of a depot of estrogen or testosterone which acts to suppress gonadotrophin release until sometime between 75 and 110 days of age. The oil droplet which remains after neonatal treatment, vestiges of which still exist at 110 days of age, can act as a depot (61). Still, in the absence of controlled measurements of serum steroids in the mature animals and in light of the extensive literature on steroidal imprinting, the present results must be viewed as being most probably due to neonatal imprinting phenomena rather than merely steroid feedback inhibition.

B. Organ Histologies:

Having established the lack of any easily located DDT effect on body weight or organ weights at any age up to adulthood, measurements for more subtle effects were undertaken. Organ histologies were done on liver, kidney, pituitary, testis and adrenal tissues chosen at random from the groups of treated animals and from the control group. From each prepared tissue 6-8 sections were examined; the subjective observations were recorded.

Pituitary, kidney and liver tissues were indistinguishable among the groups or ages; they did not differ overtly from the appearance of the controls under the general histology conditions used for preparation and staining.

Histology of the testes for groups C and I-VI were nearly identical at any given age. Sperm production was absent in animals of 25 days of age but was active at 50, 75 and 110 days. This agreed with the findings of Gellert et al. (54) of motile sperm in adult male rats neonatally injected with 3 mg of o,p'-DDT. Subjectively, the DDT-treated groups in my experiment showed some evidence of oligospermia. This was not inconsistent with Gellert's findings since he did not do sperm counts. It did, however, reinforce the recent report of Krause _et al_. (208) who did histological counts of various testicular cell types in sections made from the testes of rats injected with massive doses of technical DDT on day 4 of life.

Estrogen treatment resulted in complete aspermia through day 75 and only minimal spermatogenesis by day 110, results which were in complete accord with a report by Maqueo and Kincl (209) on histo-morphology of rats injected with estradiol benzoate on day 5 of age. TP treatment caused a definite oligospermia until at least day 75; spermatogenesis on day 110, however, appeared normal. These observations were similar to those reported in mice by Barraclough (59).

Adrenal histology for all groups on days 50, 75 and 110 seemed qualitatively similar. At all these ages the glomerulosa layer of the cortex was well defined and histologically invariate; the medulla was well defined and highly vascular; the outer (fasicular) layer of the cortex represented 30-50% of the cortical area and consisted of fairly large cells containing lipid vacuoles; the inner (reticular) layer of the cortex appeared highly vascular with small dense cells organized into definite cords.

On day 25, however, a rather striking picture emerged. Control and testosterone-treated rats exhibited a lack of the perimedullary vascularity and cellular organization typically found later. This observation was in agreement with the early observations of Howard (94). These adrenals also showed considerably less definition of the reticular and fasicular zones - cells were uniform throughout the cortex. In contrast, the glands from animals treated with o,p'-DDT or estradiol appeared very similar to normal animals at least 25 days older. The cortex had apparently aged precociously. Subjectively, the degree of aberration on the DDT-treated animals was dose-dependent.

Initial observations, summarized in Figures 21 and 22, were confirmed by examining similar slides prepared from the remaining control, DDT and EV-treated animal tissues (Table 7). Collective DDT and control observations were compared by X^2 analysis using the most vascular 25-day old control adrenal as a comparative standard. For the slides examined p was < 0.02, implying that DDT treatment with at least some dosages changed adrenal histology at 25 days of age. Histology itself cannot imply a mode of action, but similarities between EV and DDT-treated adrenals were plain.

Recapitulating, these histological examinations demonstrate that neonatal DDT treatment did indeed have an effect on the steroidogenic endocrine tissues, altering adrenal morphology and possibly sperm production as well.

C. Serum Corticosterone:

In view of the histological findings in the adrenal, the importance of measuring adrenal function became even

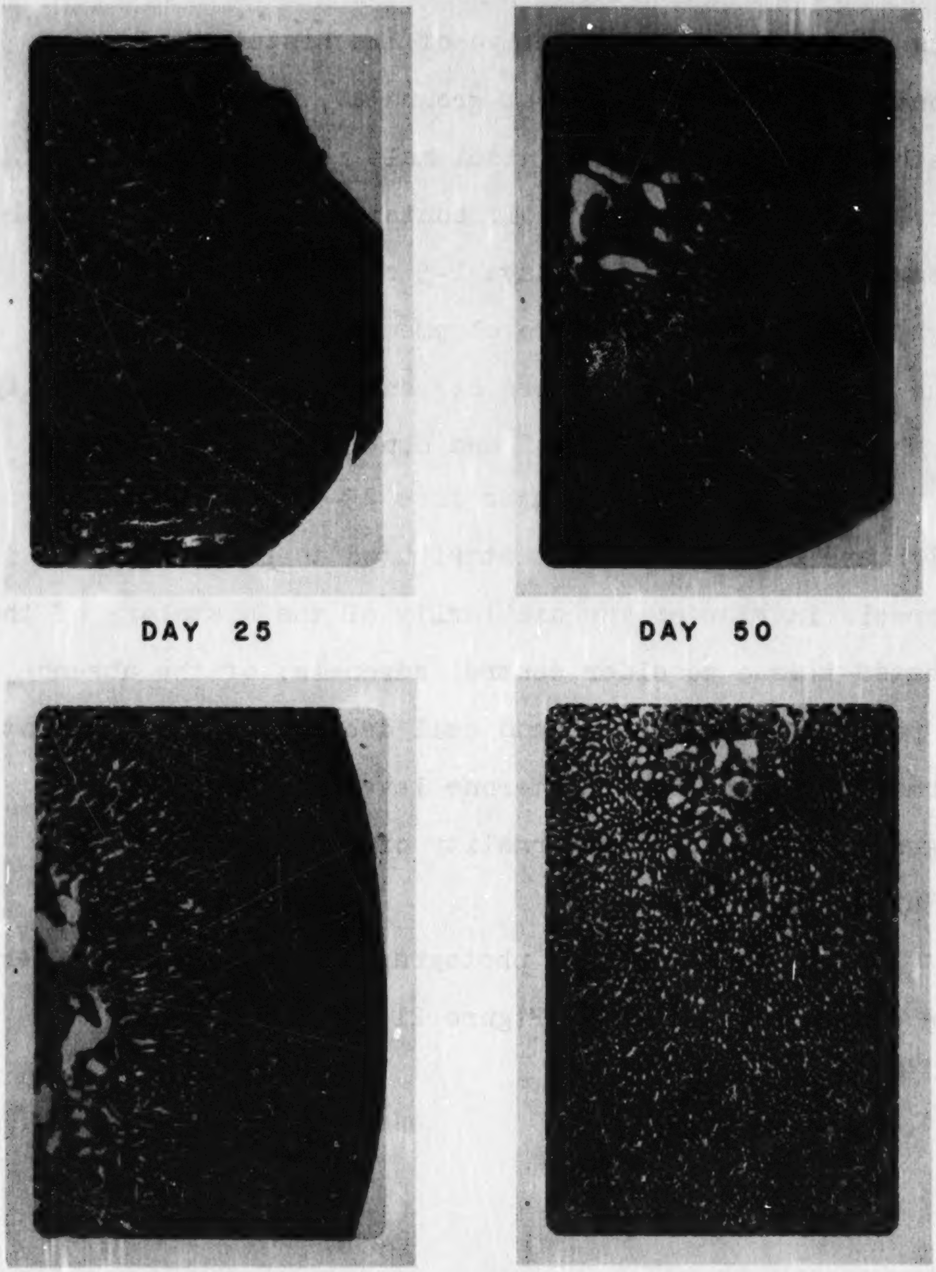
Figure 21. Normal Adrenal Development in the Male Rat: Reticular Zone Histology

These photomicrographs of normal rat adrenal glands at various ages are representative of the histological sections obtained from the control animals listed in Figures 13-20 (intact male rats injected s.c. with 0.05 ml sesame oil on each of days 1-5 of age). All photographs were taken at the same magnification (~15x) with a Zeiss polarizing photomicroscope using Kodak color photomicrography film (ASA 16; 13 DIN). The histology slides were prepared from formalin fixed tissues using paraffin embedding and hematoxylin and eosin stains; sections were saggital and 7-10 mu thick.

The medullary region of the adrenals is located at the left hand or top of the photographs and forms a distinctive border with the reticular layer of the cortex. The glomerulosa forms a densely packed region near the outer edge of the organ, except on day 110 where it is not pictured. The fasicular and reticular zones are undifferentiated at day 25, but by day 50 the organization of the vasculature and cords of the reticularis is evident. This area increases in extent at the expense of the fasiculata, at least until maturity. This gradual expansion with maturity stands in contrast to the picture seen in estrogen or o,p'-DDT treated animals (see Figure 22).

FIGURE 21

NORMAL ADRENAL DEVELOPMENT IN THE MALE RAT: RETICULAR ZONE HISTOLOGY



DAY 25

DAY 50

DAY 75

DAY 110

Figure 22. Adrenal Reticular Zone Histology at 25 Days of Age in the Neonatally Injected Male Rat

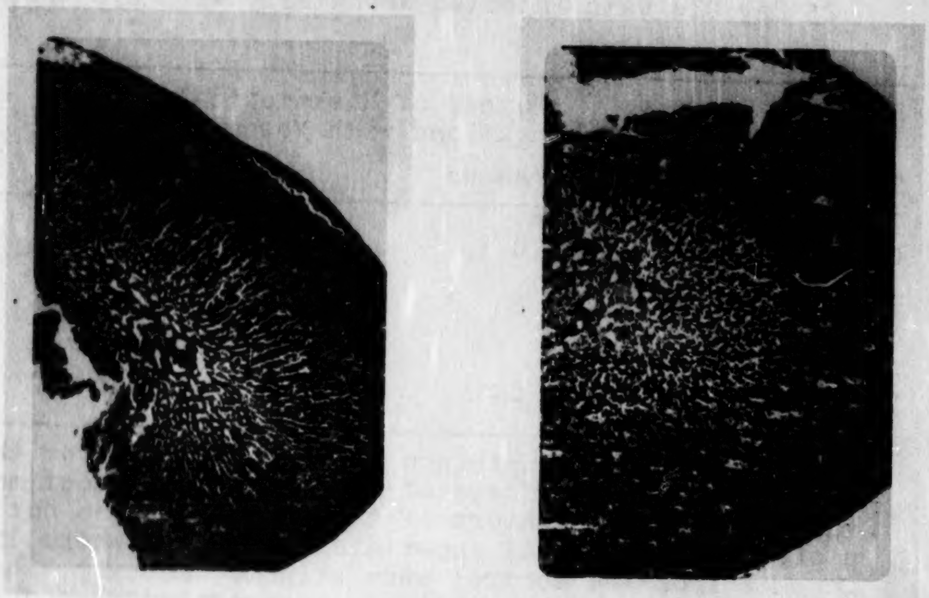
These photomicrographs of rat adrenal glands at 25 days of age are representative of the histological sections obtained from the test groups EV, II, III and IV listed in Figures 13-20 (intact male rats injected daily, s.c., with 0.05 ml sesame oil containing 1/5 of the total dosages shown on each of days 1-5 of age). They demonstrate marked precocious development of the reticular zone at this age (see Figure 21) and point to a similarity in the actions of estradiol and o,p'-DDT on this organ.

The histological changes from 25-day old controls which are evident were not attributed to perimedulary necrosis in view of the similarity of the histology of the treated tissue to older control adrenals, of the absence of polymorphonucleocytes and cell debris, of the apparent normality of the corticosterone levels secreted (see Figure 23), and of the normality of the adrenal weights obtained (see Figure 16).

Histology slides and photographs were prepared under the conditions listed in Figure 21.

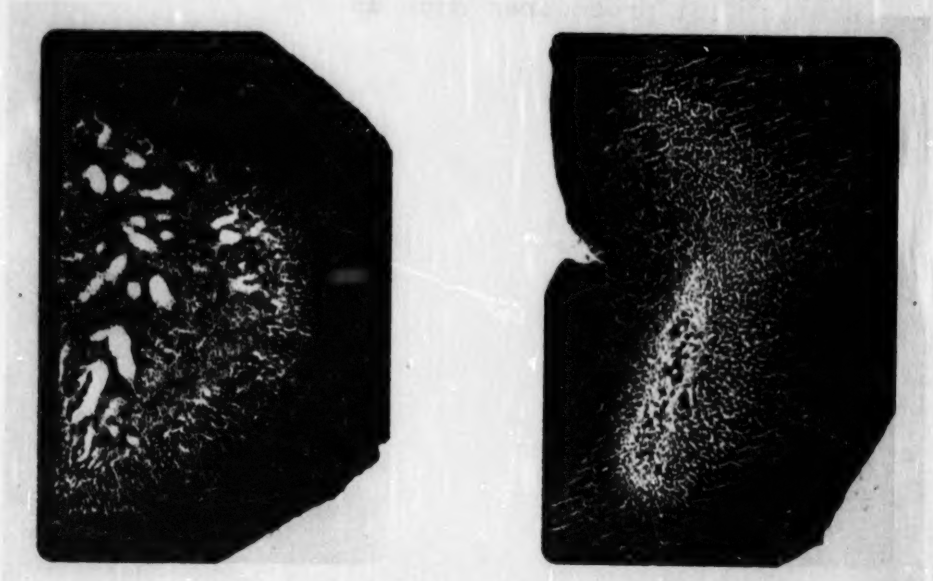
FIGURE 22

ADRENAL RETICULAR ZONE HISTOLOGY AT 25 DAYS OF AGE IN THE NEONATALLY INJECTED MALE RAT



200 UG EV

1000 UG o,p'-DDT



500 UG o,p'-DDT

100 UG o,p'-DDT

TOTAL DOSE INJECTED BY DAY 5 OF AGE

Table 7

Frequency of Increased Reticular Vascularity[1] in 25 Day Old Rats Injected Neonatally with o,p'-DDT

Treatment	Number of Histological Preparations with Vascularity Increased	Unchanged	Total
Control	0	10	10
DDT	8	12	20
Total	8	22	30

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[1] The standard for comparison was the adrenal from the control group at 25 days of age showing the most marked vascularity. The nature of the comparisons do not allow a clear indication of uncertainty in cases where the difference from the control were slight. However, these uncertainties may be partially accomodated when doing comparisons, by the use of appropriate nonparametric statistical procedures such as X^2.

more apparent than when initially planned. Many of the serum measurements had been done before the histological investigations were complete but they tended to support both the histological observations and the organ weights which had been measured. They are presented in graphical form in Figure 23.

On days 50 to 110 the mean serum levels, determined on trunk-blood taken at the time of decapitation, showed relatively small variation; control means from the rats killed at 10.00-16.00 (EST) fluctuated from 20-25 ug/100 ml of serum. Likewise, except for an occasional high value, so did the serum values from the DDT-treated groups. Testosterone treatment caused a moderate, about 10-20%, elevation of the levels on day 50 and 75, then fell to, or slightly below, the control levels on day 110. Estrogen treatment caused marked elevations above the control levels observed, ranging from a 100% elevation on day 50 to approximately a 60% elevation on day 75. However, normal levels were evident in the EV group on day 110. On day 110 the effect of illness was clearly seen in Group III as was the effect of the diurnal fluctuation of serum corticosterone in groups EV and TP; the elevated levels seen in groups II and IV-VI were also interpreted in terms of the circadian rhythm of corticosterone (210).

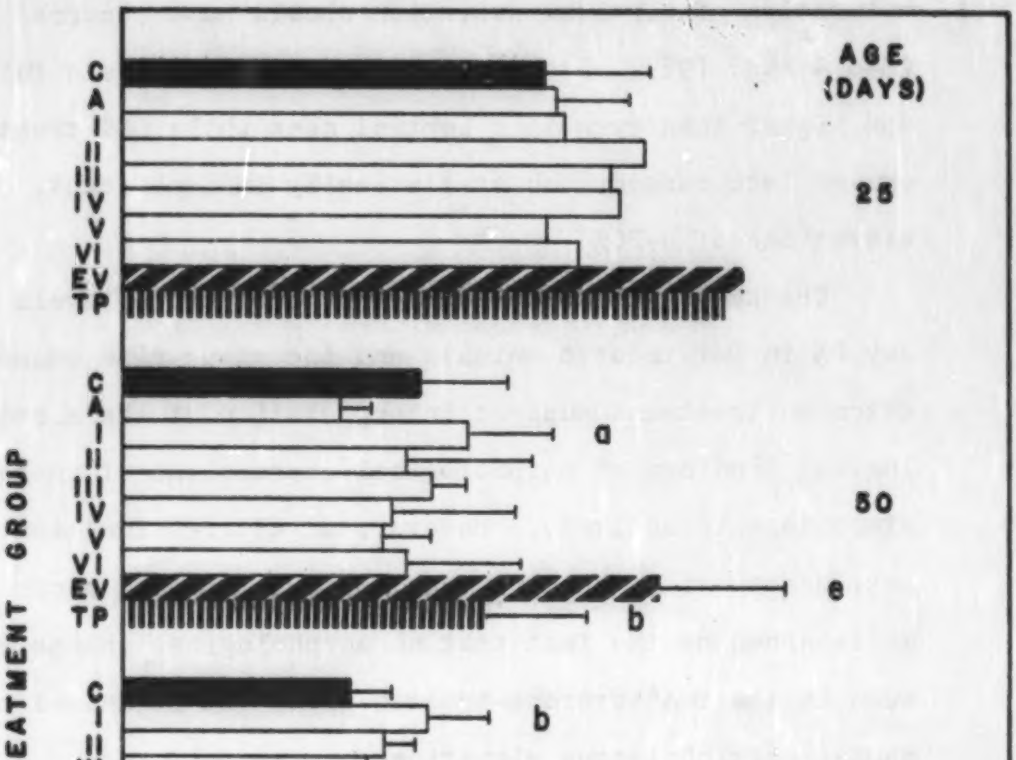
Small volumes of serum on day 25 required that samples be pooled; although multiple determinations were made on the pooled sera only the single resultant values

Figure 23. Serum Corticosterone in Neonatally Injected Male Rats

Serum corticosterone, in ug per 100 ml, as measured by sulfuric acid fluorescence is plotted versus age and treatment group. The serum samples were obtained from trunk-blood taken at the time of decapitation (12.00-16.00 (EST) except where otherwise indicated) of the animals listed in Figures 13-20 and a group of intact male rats, A, injected similarly with 5 daily doses of 2000 ug o,p'-DDT in 0.05 ml sesame oil (total dose - 10 mg) on days 1-5 of age.

Serum samples were small on day 25 and were pooled except for groups C and A. Each serum was assayed in duplicate; the means for groups were based on the means of these duplicates. Means +1 standard deviation are shown, n being 3-6 for groups A-VI and 7-12 for groups C, EV and TP. Statistically significant differences from the properly paired control groups were determined by Student's t-tests (a, $p < 0.05$; b, $p < 0.025$; e, $p < 0.0005$).

FIGURE 23
SERUM CORTICOSTERONE IN NEONATALLY INJECTED MALE RATS



were available for between-group comparisons.[4] The serum levels in weanlings in all the treatment groups were about 20-75% higher than those seen at 50 days when normal maturation of cortical secretion should have occurred (5-6 weeks) (98). Steroid treatment raised levels fully 45% higher than even this control mean while DDT treatment caused less marked, and statistically insignificant, elevations of 0-20%.

The suggestion of elevated corticosterone levels on day 25 in DDT-treated animals and the clear rise caused by estrogen treatment support interpretation of the histological findings as morphological expressions of increased steroidogenic activity. However, an altered function need not, necessarily, be correlated with an altered morphology, as is shown by the fact that no morphological change was seen in the testosterone-treated group yet it showed a marked corticosterone elevation.

The visual suggestion of some correlation between DDT dose and serum corticosterone level was evident at both 50 and 75 days of age in groups I-VI. Therefore, a series of linear regression analyses were done on the results summarized in Figure 24. If all the values were included in the calculations, either linear regression or

---

[4] This was true with two exceptions in which samples were not pooled: 1) the control group formed several serum pools; and 2) a group of males, group A, which was obtained from a small experiment conducted later but included in the overall calculations and which was neonatally injected with a total of 10 mg of o,p'-DDT.

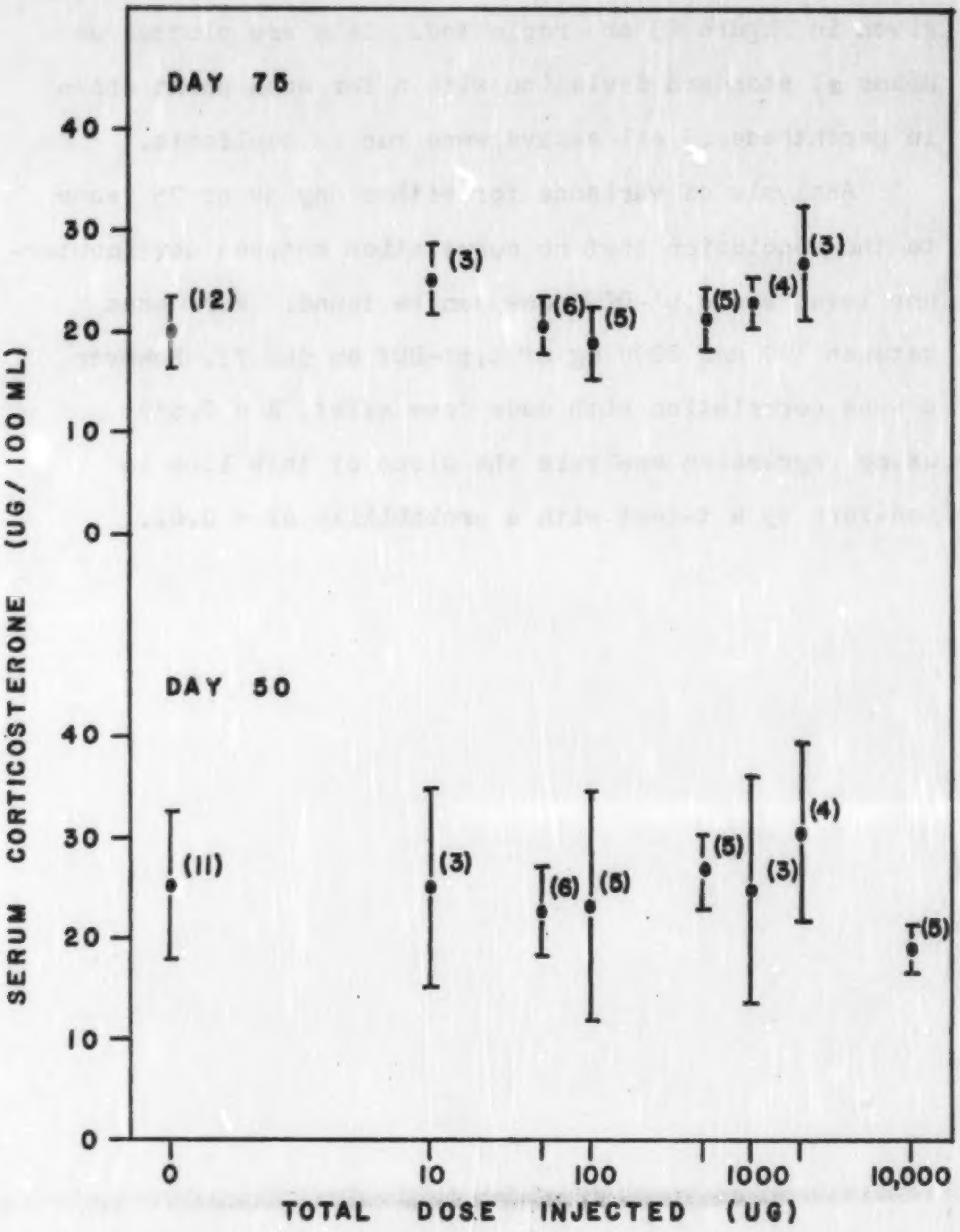
Figure 24. Serum Corticosterone in 50 and 75 Day Old Male Rats Versus Dose of Neonatally Injected o,p'-DDT

The data from groups C, A and I-VI on days 50 and 75 given in Figure 23 are replotted. Data are plotted as means ±1 standard deviation with n for each point shown in parentheses. All assays were run in duplicate.

Analysis of variance for either day 50 or 75 leads to the conclusion that no correlation between corticosterone level and o,p'-DDT dose can be found. For doses between 100 and 2000 ug of o,p'-DDT on day 75, however, a weak correlation with dose does exist, $R = 0.637$; using regression analysis the slope of this line is non-zero by a t-test with a probability of < 0.01.

FIGURE 24

SERUM CORTICOSTERONE IN 50 AND 75 DAY OLD MALE RATS VERSUS DOSE OF NEONATALLY INJECTED o,p'-DDT



ANOVA led to the conclusion that no alteration from control levels existed at either 50 or 75 days of age. However, if low and very high dose levels were eliminated from the analysis at 75 days of age a weak correlation with dose appeared, R = 0.637. This regression line allowed extrapolation of the o,p'-DDT results to the serum corticosterone levels shown by treatment with 200 ug of EV and, subsequently, to an estimation of estrogenic potency. The calculations showed that 1 ug of EV was equivalent to approximately 2,500 ug of o,p'-DDT on day 75. This estimate was well within an order of magnitude of the 1/10,000 ratio calculated by Cecil et al. (147) using a uterine glycogen-response bioassay.

The interpretation, that no dose-response effect existed, was probably true, particularly on day 50, in view of the large standard deviations which existed in what was a slightly immature and presumably unstable system. But the possibility that a multiphasic dose-response existed and was manifested by a weak dose-response relationship only in the intermediate dose range should not be ignored. DDT generates what may be antagonistic responses in the liver (microsomal induction (11-15)) and in estrogen-sensitive tissues (estrogen mimetic (22,24,26,27)). The thresholds for expression of these two effects are different (15,22) so the possibility that low levels of o,p'-DDT (0-50 ug) stimulated corticosterone breakdown and led to a decrease in serum corticosterone levels appears reasonable. Because the balance exists, it also seems reasonable that further increments (100 ug - 2 mg) of o,p'-DDT dosage could have favored increased serum corticosterone levels by an estrogen-like stimulation of the adrenals and/or an increased catabolism of circulating androgens. Even further increments (10 mg) of DDT dosage may have caused breakdown of the active form(s) of o,p'-DDT by further increasing microsomal metabolism and/or upset the balance between adrenal output and hepatic corticosterone catabolism; either mechanism would result in a drop in serum corticosterone levels. It is therefore neither impossible nor unrealistic to think that the weak correlation between dose and serum corticosterone which held for intermediate dosages of o,p'-DDT at 75 days of age might indicate an action of o,p'-DDT. Obviously, only further investigations concerning these possibilities will clarify the meaning of these results.

D. Serum LH:

Earlier studies by Rybakova (28,29) on adult rats chronically fed low levels of technical DDT uncovered a stimulatory action on the pituitary gonadotrophs, which were examined histologically, and on the pituitary content of LH, which was measured by bioassay. Gellert *et al.*, working on the female rat, had also shown that serum LH could be suppressed by o,p'-DDT in adult rats (150) and that the LH rise in response to adult ovariectomy could be blunted (54). Since the precedents existed for an effect of o,p'-DDT on hypothalamo-hypophysial function, determinations of serum LH were conducted concurrently with my examinations of tissue histology and serum corticosterone. A summary of these measurements is given in Figure 25.

The highly variable LH titers reported for immature male rats (77,82,83) were evident in control animals on day 25 and, to a somewhat lesser extent, on day 50. By day 75 the mean control level had stabilized near the lower reliable limit of the RIA, 10 ng B-640/ml. DDT-treated rats also showed highly variable levels on days 25 and 50. But, in contrast to the controls, only group VI, the lowest dosage group, had approached stability on day 50. On days 75 and 110 the levels of all the groups were statistically indistinguishable from the controls. On day 110 the high value for group III was probably again a result of illness (35,36).

Estrogen treatment caused a uniform suppression of serum LH to essentially nonmeasurable levels until day 75. A slight rise was evident by day 110. This rise may be what was reflected in the movement of several of the normalized organ weights toward control values at this time. Testosterone treatment similarly suppressed LH levels on day 25 but it apparently had lost its influence on tonic LH secretion after day 25 since an essentially

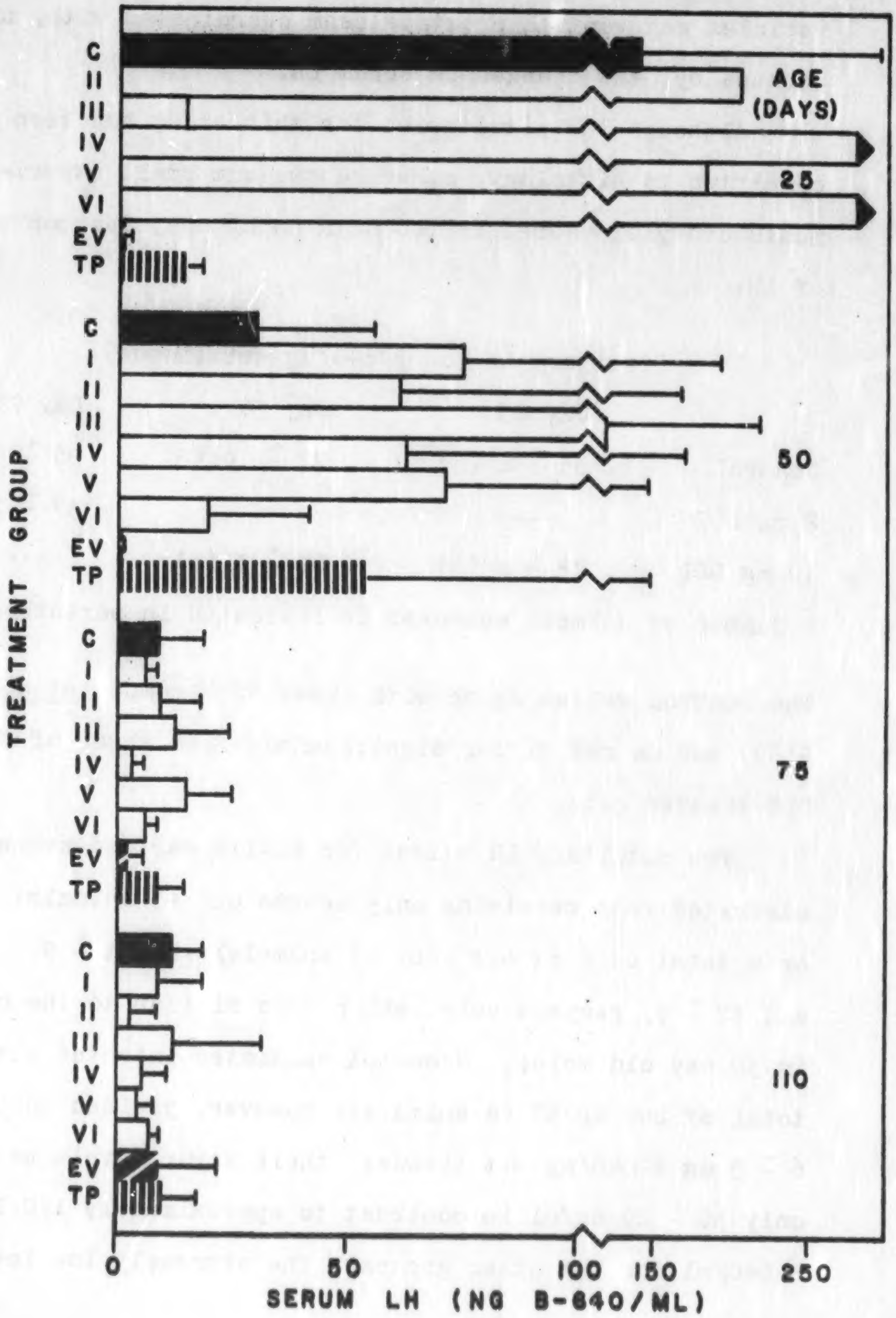
Figure 25. Serum LH in Neonatally Injected Male Rats

Levels of serum LH, in ng B-640 per ml, are shown as functions of age and dose of o,p'-DDT or steroid. Serum samples are those obtained from trunk-blood at decapitation of the animals shown in Figures 13-20. (They are the same samples used to determine serum corticosterone - Figures 23 and 24). Radioimmunoassays, using the method of Niswender et al. (169), were done at least in duplicate for each serum sample. The mean of the duplicates forms the basis for the group means (+1 standard deviation) shown; pooled samples were used for groups II-VI on day 25 of age, otherwise, n = 3-6 for groups I-VI and 7-12 for groups C, EV and TP (B-640 = 0.03 x NIH-LH-S1 by OAAD).

The large group variances at any given age mask any statistically significant differences in group means. However, two general trends are evident: 1) the decrease from generally high levels during immaturity toward low levels in maturity (75 and 110 days) and 2) the uniformly low levels of serum LH in the estradiol-treated animals.

FIGURE 25

SERUM LH IN NEONATALLY INJECTED MALE RATS



normal pattern ensued. Again, as was the case with group EV, organ weights such as testes, adrenals, seminal vesicles and ventral prostate seem coordinated with, and perhaps by, the changes in serum LH.

Although the pituitaries for this study had been committed to histology, other subsequent small experiments did yield some limited data on hypophysial content of LH:

Pituitary LH (ug B-640/mg wet tissue)

	Day 25	Day 50	Day 75
Control	35 ± 2 (3)*	77 ± 10 (3)	36 ± 8 (3)
2 mg DDT	---	---	37 ± 11 (3)
10 mg DDT	27 ± 8 (3)	69 ± 5 (5)	---

* Number of animals measured is indicated in parentheses.

The control values agree with those of Yamamoto *et al*. (180) and do not differ significantly from those of the DDT-treated rats.

The pituitary LH values for 81-110 day old neonatally castrated rats receiving only sesame oil (5 animals) or a total of 2 mg o,p'-DDT (7 animals) were 61 ± 9 and 52 ± 9, respectively, which were similar to the content in 50 day old males. Neonatal castrates injected with a total of 200 ug EV (6 animals), however, yielded only 6 ± 3 ug B-640/mg wet tissue; their serum levels were only 50 ± 20 ng/ml in contrast to approximately 1.0-1.3 ug B-640/ml for the other groups. The extremely low level of pituitary LH in the EV group easily explains the serum level. If deductive reasoning can be applied to the absence of serum LH in intact estrogenized animals from group EV of experiment 2, the result in castrates implies a similar low pituitary content in the intact rats, a situation actually found in females by Arai (211) using OAAD assays. Either of these situations can be explained by the continued presence of EV until at least 80-100 days of age. Although such a conclusion is possible on the basis of Gorski's findings with TP (61), it would not be in accord with the findings of Schiavi (207) in the pituitary. Schiavi found levels only slightly suppressed in 60 day old intact rats treated on day 5 with 100 ug of 17β-estradiol-17-benzoate and a two-fold elevation of pituitary LH content above controls at 180 days of age. Since the same vehicle was used in both Schiavi's experiments and my own the only simple explanations for the differences reside in the timing, size and form of the dose and the total volume in which it was dissolved. Each of these factors would, from what is known about imprinting (57-61) tend to favor a less drastic and prolonged effect in the experiments of Schiavi. Whether the reason is prolonged presence or imprinting, neonatal estrogen markedly affected LH in both the serum and pituitary of neonatally treated male rats for extremely long periods of time.

In summary, this second experiment affirmatively answered the initial experimental query; o,p'-DDT did indeed demonstrate an effect on the steroidogenic endocrines - it altered the early histological appearance of the adrenal cortex and possibly its function as well. Hints of other possible changes involving spermatogenesis and the production and/or release of LH at 50 days of age were found. The effects of testosterone propionate and estradiol valerinate were reexamined in a coordinated series of measurements not used before. Results stemming mostly from the estrogen-treated animals shed some doubt on the idea that estrogen esters given in oil act directly for only a short period of time after which their effects are manifestations of steroidal imprinting. This does not imply, however, that imprinting may, or does, not occur since too many experiments using other more soluble forms of the steroid or more easily absorbed vehicles substantiate the initial findings of Pfeiffer (164). Rather, the question posed is whether or not sesame oil may be a problematic vehicle in endocrinologic or screening studies.

III. Serum LH Response to Adult Castration:

While some data had been generated on the existence of an effect on the immature adrenal gland, the total absence of an effect on the hypothalamo-hypophysial-gonadal system of male rats did not seem reasonable in view of the observations of Rybakova (28,29), Gellert et al. (53,54, 150,151) and Krause et al. (208). To test the possibility that such an effect did exist but was only apparent under stressed or competitive situations, a measurement of serum LH response to adult castration was undertaken.

Six litters of male pups were divided among 4 treatment groups. Two litters received injections of sesame oil, 0.05 ml s.c. daily, on days 1-5 of age; 4 litters received 5 daily doses of 2 mg o,p'-DDT in 0.05 ml sesame oil, a total dose of 10 mg. Two of the DDT-treated litters also received other drugs; one litter received 10 equal i.p. doses of 100 ug sodium phenobarbital (PB) in 0.05 ml saline spaced 12 hours apart over the first 5 days of life while the other received 5 daily i.p. injections of 125 ug SKF-525A in 0.05 ml saline. The latter two groups were included to determine if hepatic activation played any role in mediating the effects of neonatally administered o,p'-DDT.

After allowing the animals to mature until 57 days of age 4 animals from each of the 4 groups were chosen for the experiment. Two sample bleedings (TVBE) were done on days 57 and 59 to establish a baseline. Castration was performed on day 60 and serum samples were taken at intervals until 14 days after castration, by which time serum LH levels should have begun to plateau (180). On day 14 an s.c. injection of 0.75 mg/kg of testosterone was given 6 hours prior to bleeding. The dose was small enough not to drive LH levels to baseline but large enough to ascertain if the negative feedback loop of the animal was functional (170). On day 15 after castration the animals were anesthesized lightly with ether and decapitated; trunk-blood samples were collected for LH measurement to determine if steroid clearance of the injected testosterone and the rebound of serum LH were normal. After the RIA was completed the group means were calculated for each day. They were plotted versus time after castration and submitted to 2-way ANOVA (212,213).

Results for the control and DDT-treated groups are plotted in Figure 26, those for the PB + DDT and SKF-525A + DDT groups are plotted in Figure 27. The analysis of variance, which covers days 1-11 after castration (the period after castration but before steroid treatment), is summarized in Table 8.

The expected hyperbolic rise in serum LH was seen in all four groups as was a dip of 25-40% subsequent to testosterone injection and a rise back to pre-testosterone levels. It was concluded that all groups possessed a functional feedback system. However, the plateau of the serum LH levels seen in the control and DDT-treated groups were distinctly, and statistically, different. The DDT animals reached a plateau somewhat earlier at a level fully 30% below the controls. This corresponded to a p value of < 0.005 for an ANOVA which included only the control and DDT groups (this ANOVA is not shown).

Both drug treated groups, SKF-525A + DDT and PB + DDT, had patterns which fell between the controls and the DDT-treated group. The pattern of the hepatically induced

Figure 26. Response of Serum LH to Adult Castration in Neonatally Injected Male Rats

Serum levels of LH, given in ng B-640 per ml, are plotted versus time after adult castration for groups of rats which were injected daily, s.c., on days 1-5 after birth with either 0.05 ml sesame oil (Control, o-o) or 2 mg o,p'-DDT in 0.05 ml sesame oil (DDT, x-x). Bleedings were done at 15.00-18.00 (EST) by tail-vein under light ether anesthesia, the same animals being used throughout the study. Castration was performed on day 60 of age after 2 baseline bleedings. No treatment other than bleeding occurred until 14 days after castration when hypothalamic feedback control was tested by injecting 0.75 mg/kg testosterone (T) s.c. in sesame oil 6 hours prior to bleeding. Bleeding on day 15 was without further treatment. The serum collected was assayed, at least in duplicate, by the radioimmunoassay procedure of Niswender et al. (169). The results shown are means, ±1 standard deviation, for groups of 4 animals, except for the controls on days 14 and 15 in which cases n = 3 and 2, respectively. Analysis of variance for all of the values indicate a 95% probability that the control group values were, in fact, higher throughout the experiment; a similar analysis indicates that, for the days after castration and before treatment with testosterone, the chance occurrence of the differences seen has a probability of < 0.005. Table 8 shows the 2-way ANOVA for these values and those of Figure 27.

FIGURE 26

RESPONSE OF SERUM LH TO ADULT CASTRATION IN NEONATALLY INJECTED MALE RATS

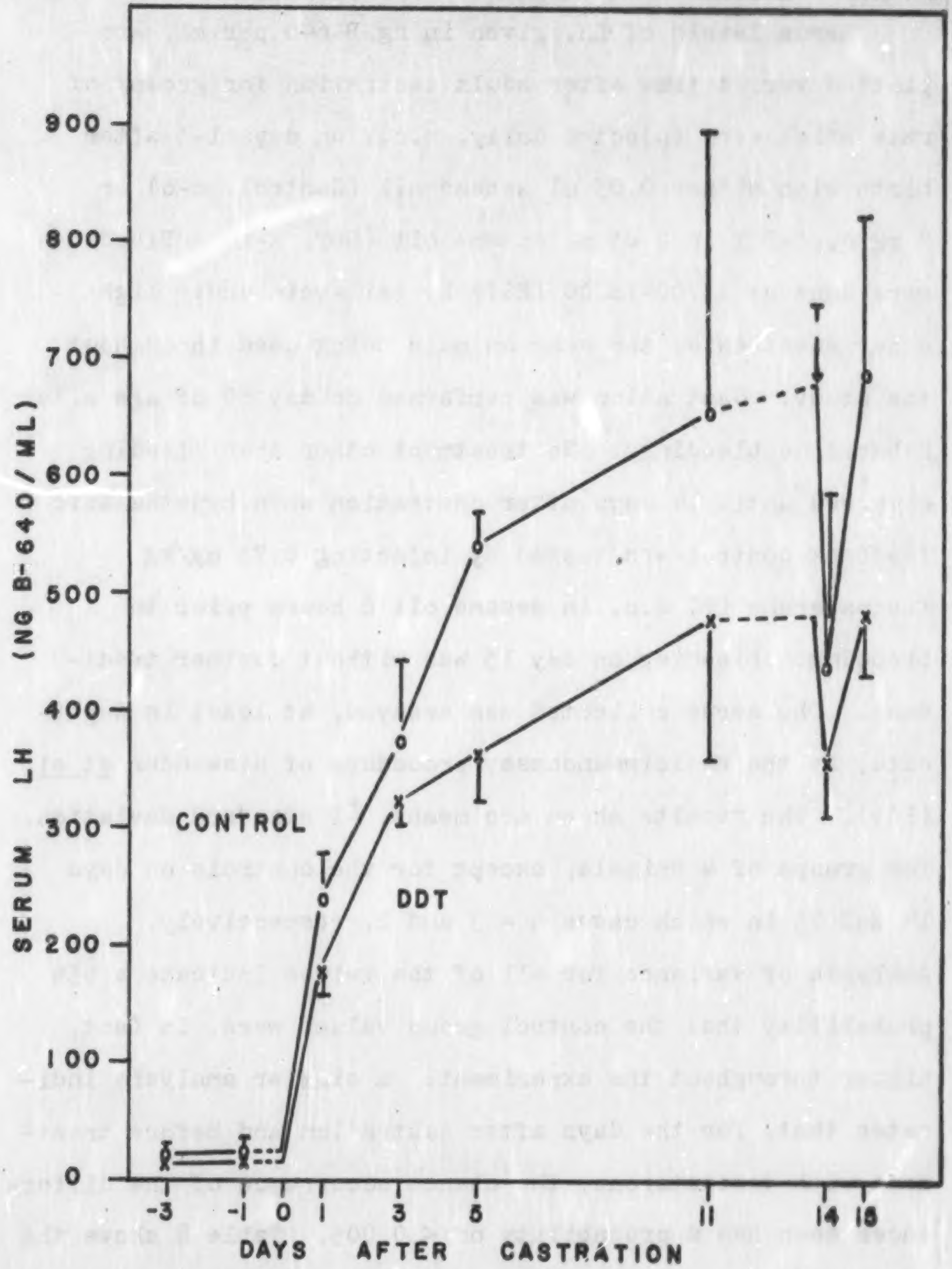


Figure 27. Response of Serum LH to Adult Castration in Neonatally Injected Male Rats

Serum levels of LH, given in ng B-460 per ml, are plotted versus time after adult castration for groups of rats which were treated on days 1-5 of age as follows: group SKF-525A + DDT (▼-▼) was injected daily with 2 mg o,p'-DDT in 0.05 ml sesame oil, s.c., and 125 ug (25 mg/kg) of SKF-525A in 0.05 ml neutral saline, i.p.; group phenobarbital + DDT (●-●) was injected daily with 2 mg o,p'-DDT in 0.05 ml sesame oil, s.c., and 2 doses - 12 hours apart - of 100 ug (40 mg/kg) sodium phenobarbital in neutral saline, i.p. Bleedings, castration, testosterone treatment and sample assays were the same as described in Figure 26.

The results shown are means, ±1 standard deviation for groups of 4 animals. All values were examined by analysis of variance simultaneously with the groups shown in Figure 26. Comparisons of the SKF-525A + DDT and/or phenobarbital + DDT groups with the control group (Figure 26) over the entire test period or over the period after castration and prior to treatment with testosterone indicate no significant differences between these groups (as determined by Tukey's test for multiple comparisons (214)).

FIGURE 27

RESPONSE OF SERUM LH TO ADULT CASTRATION IN NEONATALLY INJECTED MALE RATS

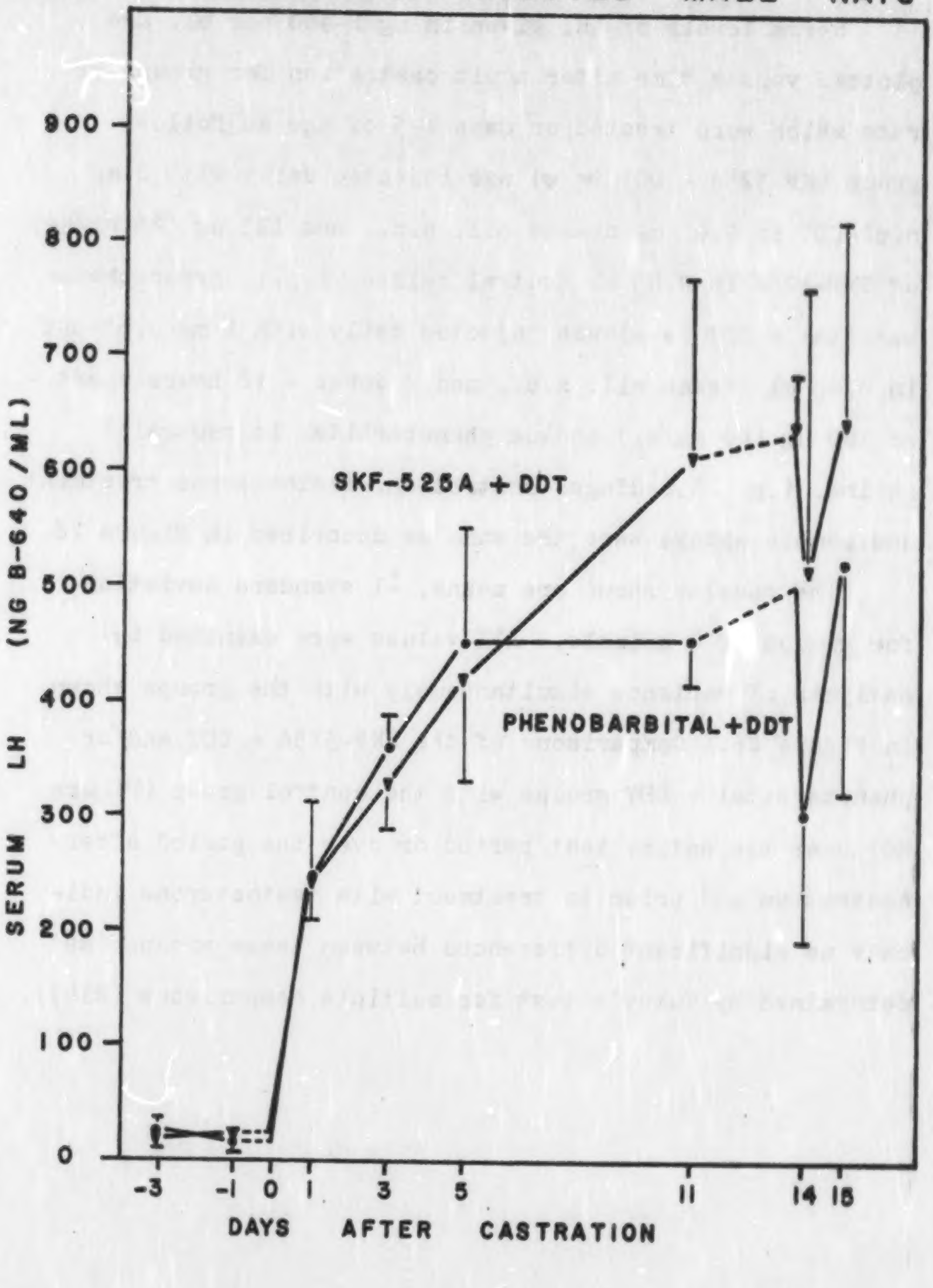


Table 8

Two-Way Analysis of Variance for Castration Response Data from Adult, Neonatally Injected Male Rats: Analysis of Days After Castration and Before Testosterone Administration

Treatment	Raw Data from Figures 26 and 27 (LH in ng B-640/ml Serum)					
	Day					
	1	3	5	11	Σx_i	Σx_i^2
Control	180.72	382.09	543.76	702.58	7,190.72	3,833,909.77
	240.74	323.72	518.11	435.10		
	233.54	315.40	579.29	972.29		
	285.25	470.43	511.26	496.14		
o,p'-DDT	210.98	343.78	394.95	373.95	5,382.56	2,052,224.82
	155.18	295.99	311.08	425.95		
	172.65	336.16	349.40	465.07		
	173.00	315.30	402.25	656.87		
o,p'-DDT + SKF-525A	250.17	289.34	312.60	696.10	6,341.33	2,919,215.24
	226.26	330.84	441.90	484.66		
	270.06	367.22	510.74	788.93		
	209.88	201.95	390.58	470.10		
o,p'-DDT + PB	260.49	331.72	575.83	489.70	6,063.25	2,472,126.38
	278.75	392.79	409.13	412.20		
	139.33	381.01	327.74	464.79		
	292.38	358.00	499.39	450.00		
Σx_i	3,579.38	5,535.74	7,078.31	8,784.43	24,977.86	

Table 8 (Cont.)

$\sum x_i^2$	836,070.53	1,946,633.19	3,260,199.02	5,234,573.47	11,277,476.20

$$(\sum x_i)^2 = (24,977.86)^2 = 623,893,490.1$$

$$\sum_1^{N_b}(\sum_1^4 x_i)^2 = (940.25)^2 + (1491.64)^2 + (2152.72)^2 + (2606.11)^2 + (711.81)^2 + (1291.23)^2 + (1457.68)^2 + (1921.84)^2 + (956.37)^2 + (1289.35)^2 + (1655.82)^2 + (2439.79)^2 + (970.95)^2 + (1463.52)^2 + (1812.09)^2 + (1816.69)^2 = 43,467,370.57$$

$$\sum^{\text{Day}} (\sum^{R_x} X)^2 = (7190.72)^2 + (5382.56)^2 + (6341.33)^2 + (6063.25)^2 = 157,653,873.0$$

$$\sum^{R_x} (\sum^{\text{Day}} X)^2 = (3579.38)^2 + (5535.74)^2 + (7078.31)^2 + (8784.43)^2 = 170,725,061.4$$

Summary Computations

Source of Variation	Amount (SS)	D.F.	Mean Variance (MS)	F versus Error	Occurrence by Chance
Treatment (R_x)	105,031.28	3	35,010.43	4.0925	< 0.025
Day after Castration	921,980.55	3	307,326.85	35.9242	< 0.0005
Interaction	91,495.03	9	10,166.11	1.1883	> 0.05
Error	410,633.56	48	8,554.87	---	---
Total	1,529,140.42	63			

$N_b = 16$ = Number of boxes, i.e. groups of measurements with the same treatment on the same day.

group, PB + DDT, tended to resemble the DDT hyperbola more while the SKF-525A + DDT, or hepatically inhibited, group favored the control pattern. Neither of these groups differed significantly from the control group or from each other. A set of pairwise analyses (ANOVA) did show that the SKF-525A + DDT group was different from the DDT group ($p < 0.05$) and that the PB + DDT group approached statistical difference from both control and DDT-treated groups ($0.05 < p < 0.10$).

The analysis of variance including all four treatment groups, Table 8, demonstrated the anticipated effect of time after castration by giving it a p value of < 0.0005, i.e., time after castration contributed significantly to the overall variance of the results from the overall (grand) mean of the results. Treatment also showed a significant effect, $p < 0.025$. Time after castration and treatment were shown to be independent when they exhibited no interaction, $p > 0.05$. Tukey's (honestly significant difference) tests (214) were performed for each of the possible data contrasts within the data set but only one reached significance at the 5% level (Control - DDT). The value of the (Control - (PB + DDT)) contrast approached, but did not reach significance. A simple estimation of F for the maximum and minimum cell variances indicated that the data had fulfilled the requirement for homoscedasticity and that the use of ANOVA was fully justified.

The observed similarity of the SKF-525A + DDT group and the controls implied that SKF-525A had antagonized whatever action of o,p'-DDT had resulted in making the DDT-treated group different from the controls. Because SKF-525A acts as an inhibitor of microsomal function and can presumably block DDT breakdown in mammals as it does in insects (107,215), the amelioration of the DDT effect on the postcastration rise in LH implied that an active metabolite of o,p'-DDT was involved in producing the LH suppression seen in the DDT-treated group. Further support for this conclusion was provided by the position of the PB + DDT curve. Phenobarbital is a known inducer of DDT metabolism in the rat (175) as is DDT itself (115). The net result of injection of both PB and DDT could be either a more rapid formation of an active DDT metabolite than occurs with DDT treatment alone or a more rapid inactivation of DDT or its metabolite(s) than occurs with DDT alone. The first alternative should potentiate any observed actions of DDT while the second should alleviate them. Since the PB + DDT curve fell between that of DDT and SKF-525A + DDT, a balance between the two alternatives appeared to have occurred.

Other measurements on these animals indicated their similarities. Variables such as organ weights and pituitary LH content (144 ± 12 ug B-640/mg wet weight) did not differ significantly between the control, DDT and drug treated groups nor did they differ from published values (180,207).

To summarize, a second experiment has indicated the existence of an effect of o,p'-DDT on the steroidogenic endocrine system of the male rat. This experiment has shown the occurrence in male rats neonatally treated with o,p'-DDT of the same blunting of the LH response to adult castration which Gellert et al. (54) demonstrated in the neonatally injected female rat. At the o,p'-DDT dose used the decrease of 30%, observed at 11 days postcastration, agrees well with the 25% diminution seen in the female at 3 weeks postovariectomy. In either sex the difference between controls and the DDT-treated animals may be an hypothalamic imprinting (as proposed by Gellert et al.), a peripherally mediated change of gonadotrophin or steroid metabolism, or an alteration of hypothalamic, pituitary or gonadal hormone sensitivity caused by occupation of steroid receptors by o,p'-DDT or one of its metabolites. The indication that a metabolite might be involved is seen in the present results in the similarity of the responses of the hepatically-induced, DDT-treated group (PB + DDT) and the DDT-treated group. It is also seen in the similarity of the responses of the hepatically inhibited, DDT-treated group (SKF-525A + DDT) and the control group. These results reinforce the hypotheses of an active o,p'-DDT metabolite put forward by several authors (22,146, 147) to explain such phenomena as the inhibition of the uterotropic action of o,p'-DDT by pretreatment with the microsomal poison carbon tetrachloride (22).

CHAPTER 4

EXPERIMENTS ON THE EFFECTS OF ADMINISTERING o,p'-DDT TO NEONATAL MALE RATS VIA THEIR MOTHER'S MILK

The last chapter dealt with experiments in animals directly injected with doses of o,p'-DDT. This chapter is a description of the dosage of neonatal rats with o,p'-DDT via their dams and some of the results of such dosage. Since most of these experiments were conducted concurrently with those described in Chapter 3 and some used similar designs and methodology, they do not represent advanced experiments but describe results obtained by using a different route of exposure.

I. Administration of o,p'-DDT to Neonatal Rats Via the Dam:

Since the natural route of exposure to o,p'-DDT, or other synthetic chemicals, during the perinatal period is via the placenta and/or mother's milk, and since many materials, including DDT, have been shown to be passed via those routes (55,56,153,187), it appeared expedient to ascertain the effects of o,p'-DDT when administered to the pup via such a natural route. Therefore, after delivery and assignment of pups to litters of the needed sizes (6 to 12 per dam) the dams were given daily i.p. injections, until weaning, of either 0.1 ml of DMSO or the same volume containing 50 mg of o,p'-DDT dissolved in DMSO. The daily dose of o,p'-DDT chosen was 1/10 of an acute LD_{50}.

Body weights for both control and DDT-injected dams were monitored for one group of dams; growth of the pups was also measured. Control and DDT-treated dams showed similar weight profiles through weaning as did their pups. Growth of the litters was similar to the sesame oil injected controls used in other experiments and to the untreated litters of similar size described by Ošťádalová et al. (163,216). Other than local discomfort immediately (0-15 minutes) following injection, the dams did not show any noticeable untoward effects.

The transfer of o,p'-DDT from dams fed diets containing 20 or 200 ppm of technical DDT to their pups was implied in a study (187) of the concentration of DDT metabolites in the milk curds found in the stomachs of suckling pups. In that study Ottoboni and Ferguson demonstrated a DDT concentration in milk lipid ranging from 3 to 10 times the concentration in the dams' feed; the concentration ratio (milk/feed) decreased with increased DDT concentration in the feed. The study did not, however, indicate the amount of the DDT dose absorbed by the pups. Because that study was based on data generated by long term feeding, because it did not measure DDT uptake by the pups and because it included feeding during pregnancy it could only serve as a general model for an investigation specifically designed to determine what dose of o,p'-DDT and/or its metabolites were absorbed by pups suckling an injected dam. The present experiment measured both the secretion of o,p'-DDT and its neutral metabolites and their absorption by suckling pups.

On the day of parturition pups were sorted into litters of 6 male pups each and the daily injections of DMSO or DDT into the dams were begun. The next day, day 1, one pup from each of 3 DDT-treated and 4 control litters were removed at random, killed and the stomachs dissected out for separate storage. The pups were immediately replaced in the litters by females of similar age. The same procedure was repeated on days 3, 5, 10, 15 and 20 after birth (only 1-3 samples were collected from each treatment group after day 10). Collected samples were processed and measured by the methods described in Figures 9 and 10 (see Chapter 2, Section IV.E.). Only the curded milk in samples taken at 15 and 20 days was processed to avoid spuriously low values due to the presence of uncontaminated rat chow in the contents of the stomach. No o,p'-DDE was found in either milk curd or whole body extracts.

Group means were generated for each compound measured for each sampling day and these were plotted against time after birth (which closely approximated time after initial o,p'-DDT administration). The observed differences in analog secretion (Figure 28) and uptake (Figure 29) between control and DDT-treated groups were dramatic.

Concentration of o,p'-DDT in milk (Figure 28) rose gradually from 1.3 ppm on day 1 to 4 ppm on day 20. The

Figure 28. Secretion of DDT Analogs in Rat Milk

The concentration, in ug per g of milk curd, of various DDT analogs extracted from the curded milk removed from rat pups of various ages is shown. The pups were reared in litters of 6 pups each by dams which were injected daily, i.p., from day 0 (= birth) onward with either 0.1 ml DMSO (x-x) or 0.1 ml DMSO containing 50 mg o,p'-DDT (●-●). The curds were extracted according to the procedure shown in Figure 9 and subjected to gas chromatography under the conditions listed in Figure 10. The data shown are means, ±1 standard error, for both treatment groups; error bars do not appear for those points where the symbol covers the error interval. Each point represents 2-4 milk curds except for days 10-20 of the controls where single values are shown.

FIGURE 28

SECRETION OF DDT ANALOGS IN RAT MILK

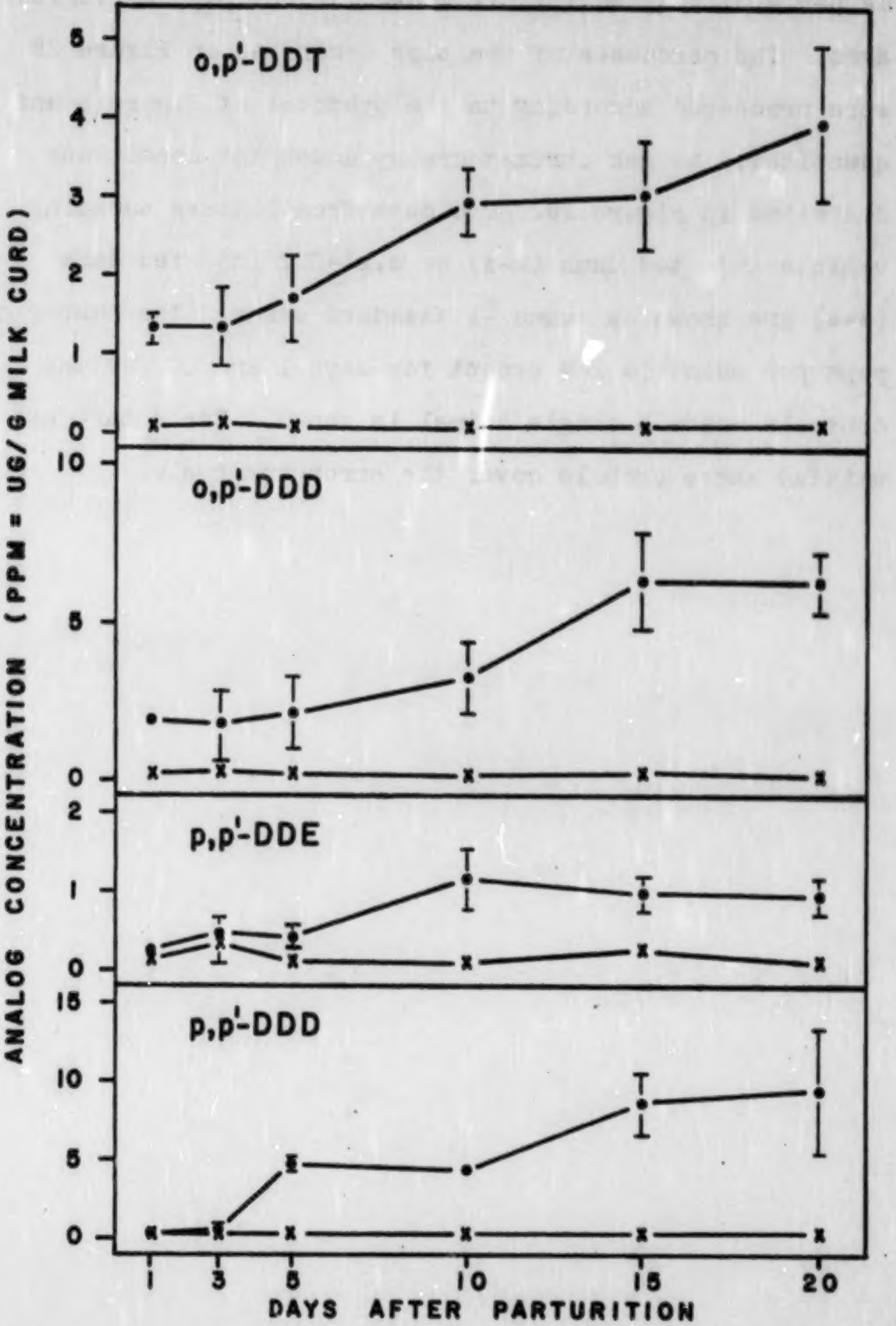
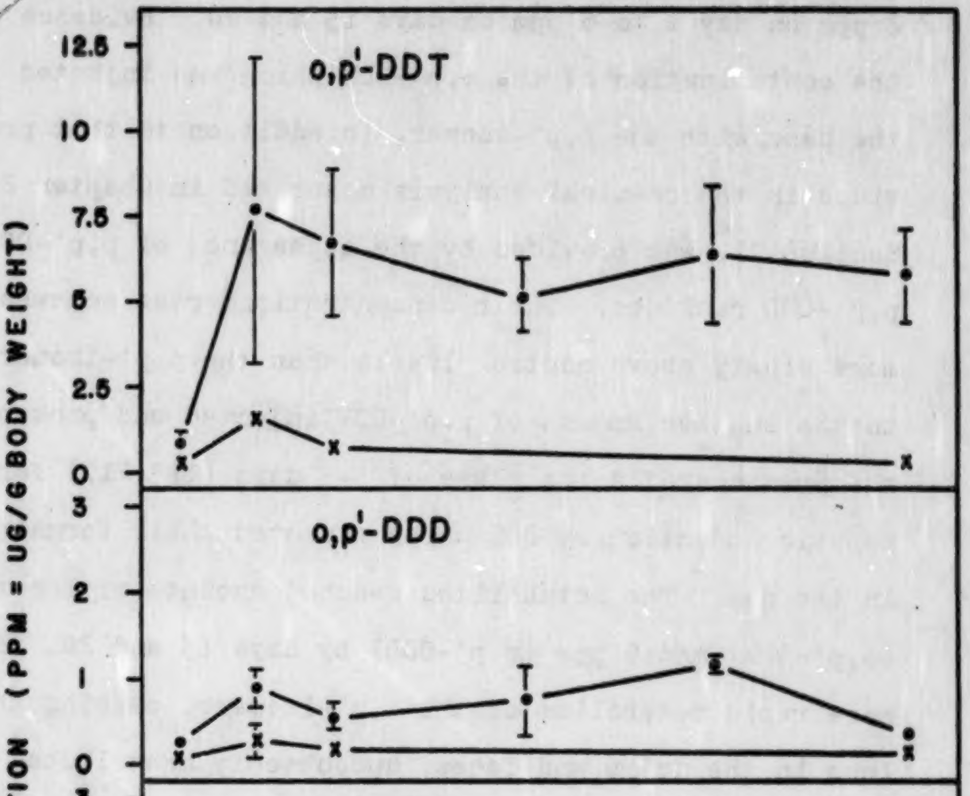


Figure 29. Uptake of DDT Analogs by Suckling Rats

The tissue concentrations of various DDT analogs, in ug per g of body weight, is shown for rat pups of various ages. The carcasses of the pups described in Figure 28 were processed according to the protocol of Figure 9 and quantitated by gas chromatography under the conditions described in Figure 10. The data from litters suckling vehicle-injected dams (x-x) or o,p'-DDT injected dams (●-●) are shown as means ±1 standard error. The number of pups per point is 2-4 except for days 1 and 20 for the controls where a single animal is shown. Error bars are omitted where symbols cover the error intervals.

FIGURE 29

UPTAKE OF DDT ANALOGS BY SUCKLING RATS



concentration of o,p'-DDD, the major neutral metabolite of o,p'-DDT, rose in a fashion parallel to o,p'-DDT from 2 ppm on day 1 to 6 ppm on days 15 and 20. Evidence of the contamination of the o,p'-DDT, which was injected into the dams, with the p,p'-isomer, in addition to that provided in the chemical analysis described in Chapter 2, Section II, was provided by the appearance of p,p'-DDE and p,p'-DDD residues. Their concentrations rose somewhat more slowly above control levels than the o,p'-isomers due to the smaller amount of p,p'-DDT injected and probably to the presence of a lag phase of 3-5 days (113,217) for the hepatic induction by DDT which promoted their formation in the dam. The metabolites reached amounts of 1 ppm (p,p'-DDE) and 9 ppm (p,p'-DDD) by days 15 and 20. The more rapid metabolism of the o,p'-isomers, causing their loss in the urine and feces, undoubtedly contributed substantially to the implied unequal retention of the p,p'-DDT isomer by the dam and to her subsequent secretion in milk of disproportionately large amounts of p,p'-DDE and p,p'-DDD.

Whole body o,p'-DDT concentrations in the pups (Figure 29) rose abruptly from control levels on day 1 to a plateau of 6-7.5 ppm by day 3. This clearly demonstrated the absorption of o,p'-DDT. If an estimation of 70% water was made for total body weight, and a similar estimation of 75-80% water was made for stomach curd weight (218), the concentrations of o,p'-DDT based on solid weights were

18 and 12-15 ppm for the whole body and curd, respectively. Thus, accumulation was at least an equilibration of the concentrations in the pups' body tissues with those in the milk.

Though the body concentrations of o,p'-DDD followed the temporal pattern of o,p'-DDT accumulation they never reached the levels found in milk, on either a total or solid weight basis. A partial answer to this discrepancy with the results found for o,p'-DDT may lie in the occurrence of on-column breakdown of o,p'-DDT to o,p'-DDD. This breakdown was encountered in an analysis of pure o,p'-DDT conducted after analysis of the body residues and the milk curd samples had been completed. A mass of 10-20% of an injection of o,p'-DDT appeared as o,p'-DDD, probably as the result of interaction with lipid residues deposited on the column near the injection port. The same problem has been described for p,p'-DDT by Burke (219). It constitutes a needed correction (increase) of less than 20% for the o,p'-DDT values in milk curd samples (with an equivalent decrease in measured o,p'-DDD levels). Analyses of pure pesticides conducted between the body residue and milk curd analyses indicated no breakdown problem. Therefore, any adjustments would apply to the milk curd results only. Possibilities which are more capable of fully explaining the difference seen in the milk and body residues of o,p'-DDD in comparison to those seen with o,p'-DDT may be the occurrences of a more active metabolism or excretion of ingested o,p'-DDD by the pups or a selective exclusion of o,p'-DDD by the gut.

Body uptake of p,p'-DDE approximates a sigmoidal curve less steep than the uptake of the o,p'-compounds. The rise to 1.5 ppm between day 10 and day 15 follows both attainment of the plateau of secretion in milk and the critical hypothalamic imprinting period. On a solid weight basis the concentrations of both tissue and milk would be near 4-5 ppm on day 15, a situation adequately described as a passive accumulation driven by a concentration gradient; just as might have been expected for a nonmetabolizable, hydrophobic compound such as p,p'-DDE.

As with body residues of o,p'-DDD, body residues of p,p'-DDD never approach the levels theoretically possible on the basis of the residues seen in milk, levels which might cause neural or reproductive toxicity (see p. 30-31) or levels approaching those of o,p'-DDT during the critical imprinting period. A possible on-column breakdown of the 0.1 ng p,p'-DDT per ul, originally added as an internal standard in milk curd analyses could only account for 1-2% of the p,p'-DDD mass found in milk and therefore is inconsequential in explaining the difference between concentrations seen in milk curds and total body extracts. Time courses for either p,p'-DDD or o,p'-DDD uptake in pups do, however, indicate that breakdown after absorption is a very active process. The abrupt fall to control levels after 15 days is not accompanied by similar falls in o,p'-DDT or p,p'-DDE as would be the case if only decreased uptake, due to introduction of solid food (163,216), was the cause. Therefore, active metabolism by pups in the presence of decreased intake of all forms of DDT must have caused the observed fall in body residues after day 15 and was probably responsible at earlier times for maintaining DDD levels below those expected by passive accumulation. Hepatic induction in pups (112,153) was not clearly indicated but may have been reflected in the dip in o,p'-DDD and o,p'-DDT values which occurred between days 3 and 10 since secretion in milk continued to rise throughout that period.

Total dosage for the period of birth through weaning was calculated by integrating a set of curves, extrapolated to 25 days of age, over time. Total administered dose (cumulative dose) was obtained from milk curd concentrations while total effective or absorbed dose (body burden) was calculated from body concentrations. Milk production was assumed to be 24.7 g/day - the mean value obtained by Hanwell and Linzell (220) for dams suckling litters of 6 pups. Mean body weights, exclusive of stomach, were used in computation.

When body burden of o,p'-DDT was plotted against cumulative dose of o,p'-DDT (Figure 30) a curve intermediate between a straight line and a hyperbola was generated. Since any curve fitted to the results lay above a line with a slope of unity and thus forced the conclusion that more o,p'-DDT was absorbed than was supplied, one of two conditions must have occurred. Either the assumed

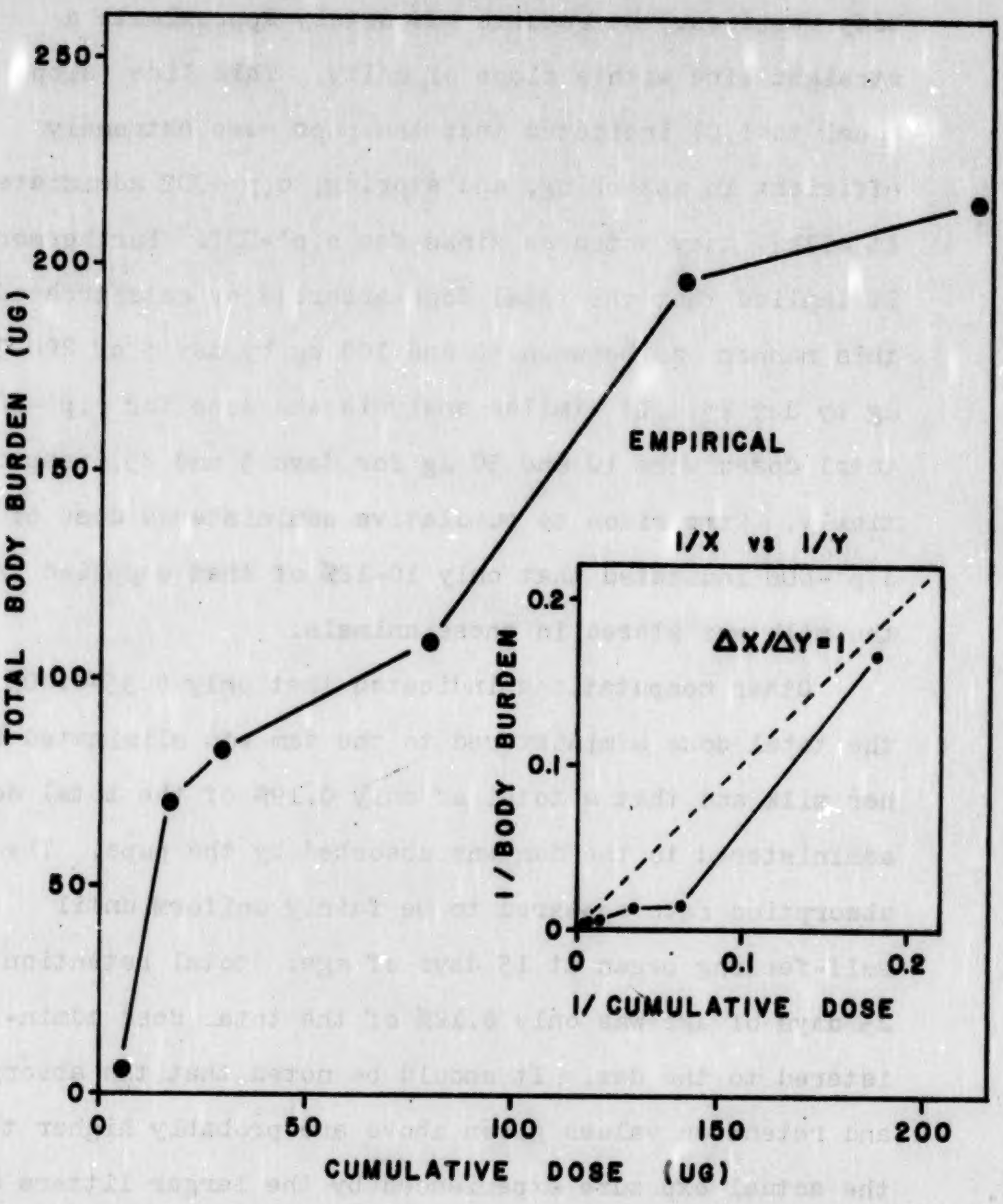
Figure 30. Uptake of o,p'-DDT by Suckling Rat Pups: Total Body Burden Versus Cumulative Dose

Total body burden, calculated as the product of o,p'-DDT tissue concentration and average body weight at the time of sacrifice, is plotted versus cumulative ingested dose of o,p'-DDT, calculated as the product of daily milk weight and milk curd concentration integrated over the age of the animals in days. Milk weight was taken as 24.7 g/day based on the findings of Hanwell and Linzell (220) for litters of 6 pups. The empirical curve was generated from the data for the DDT exposed pups described in Figures 28 and 29.

The inset shows a graph of the inverse functions with a comparison to a line $\Delta x/\Delta y = 1$, describing complete storage of ingested residues. It should be noted that the empirical lines describe systems which are storing more residues than are being ingested. If the errors for both uptake and storage measurements (see Figures 28 and 29) are considered, however, the data may be found to include the approximation of a line with a slope of 1. Thus the data appear to describe a nonsaturable system, i.e., a residue sink.

FIGURE 30

UPTAKE OF o,p'-DDT BY SUCKLING RAT PUPS: TOTAL BODY BURDEN VERSUS CUMULATIVE DOSE



production of milk was too low or the estimates of milk residues were too low. If allowances were made for a 10-20% underestimate of o,p'-DDT, caused by on-column breakdown, and for the variances of both milk curd and body residues, the results adequately approximate a straight line with a slope of unity. This line (slope equal to 1.0) indicated that the pups were extremely efficient in absorbing, and storing, o,p'-DDT administered in milk; they acted as sinks for o,p'-DDT. Furthermore, it implied that the total dose absorbed by rats treated in this manner was between 50 and 100 ug by day 5 or 200-300 ug by day 25. If similar analysis was done for o,p'-DDD, total doses were 10 and 50 ug for days 5 and 25, respectively. Comparison to cumulative administered dose of o,p'-DDD indicated that only 10-12% of that supplied in the milk was stored in these animals.

Other computations indicated that only 0.35-0.40% of the total dose administered to the dam was eliminated in her milk and that a total of only 0.19% of the total dose administered to the dam was absorbed by the pups. The absorption rate appeared to be fairly uniform until self-feeding began at 15 days of age; total retention by 25 days of age was only 0.12% of the total dose administered to the dam. It should be noted that the absorption and retention values given above are probably higher than the actual exposure experienced by the larger litters used in other experiments described later since milk production per pup falls somewhat as the litter size increases (220). Conversely, because total milk production increases with larger litters, total dose secreted increases. Therefore, this experiment only describes an upper limit of exposure per pup and a lower limit of total secretion by the female.

In summary, this experiment was designed to determine the dosage of o,p'-DDT administered to rat pups suckled by DDT-injected dams; it showed that the doses administered in the milk and absorbed by the pups were well within the dose-range examined in directly injected rats. It generated some information on the dynamics of this transfer from mother to offspring in terms of the forms and quantities involved. And, it inferred the role of hepatic induction in transformations in both the dam and pup. Finally, the experiment indicated that if a "DDT-saturated" female were used as a foster dam for a portion of the neonatal or preweaning period the efficient absorption of o,p'-DDT would allow dose response experiments to be conducted by using the natural route of exposure.

II. Organ Weights in Adult Rats Neonatally Treated with o,p'-DDT Via Their Dams:

A brief experiment similar to the initial experiment on directly injected rats was performed using the injected dam as the means of exposure. Litter size averaged 8 pups. Autopsies were done at 102-138 days of age; both intact and neonatally castrated animals were examined. Growth was only examined sporatically but indicated general agreement among the groups prior to 40-50 days of age.

Final body weights (Table 9) were similar in both castrate groups but the DDT-treated intact animals were about 10-12% lighter than their vehicle-exposed controls. All body weights were indistinguishable from the corresponding groups shown in Table 6 and Figure 13.

Organ weights, normalized to body weight, demonstrated the effects of castration but did not reflect any overt changes due to treatment with o,p'-DDT. Only the testes showed a slight change in normalized weight due to the presence of DDT, but this was explained by the difference in body weights between the control and DDT-treated group; raw organ weights did not differ. The other normalized organ weights, with the exception of the pituitary, were similar to those seen in the directly injected rats described in Table 6 and in Figures 14-20.

The weight differences which exist between these findings and those obtained in the experiments done on directly injected animals (Table 6 and Figure 14), in regard to the pituitaries of both the intact and castrate groups, were not explicable on the basis of differing body weights. The difference was also not reflected in a change in pituitary LH content or serum LH levels in the castrate groups; pituitary LH content was 55 ± 7 and 55 ± 6 ug B-640/mg wet tissue for control (3 animals) and DDT-treated (3 animals) groups, respectively; serum LH levels were

Table 9

Effects of Suckling an o,p'-DDT Injected Dam[1] on Body[2] and Organ Weights of Intact and Neonatally Castrated Male Rats

Percent of Body Weight[3]	Intact Control (6)	 DDT (6)	Castrated Control (9)	 DDT (14)
	(455 ± 47)	(401 ± 29)**	(333 ± 21)	(353 ± 35)
Pituitary x 10^3	3.37 ± 0.19	3.51 ± 0.38	4.77 ± 0.68	4.27 ± 1.34
Adrenals x 10^2	1.36 ± 0.09	1.30 ± 0.06	2.05 ± 0.28	2.09 ± 0.43
Testes	0.74 ± 0.07	0.80 ± 0.05*	-----	-----
Seminal Vesicles x 1	0.33 ± 0.09	0.31 ± 0.09	-----	-----
Seminal Vesicles x 10^3	-----	-----	8.36 ± 3.36	11.37 ± 7.06**
Ventral Prostate	0.09 ± 0.02	0.11 ± 0.02	-----	-----
Liver	-----	-----	3.92 ± 0.68	3.54 ± 0.50
Kidneys	-----	-----	0.76 ± 0.15	0.71 ± 0.13

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[1] All pups were suckled prior to weaning at 25 days of age by dams who received daily i.p. injections of 50 mg o,p'-DDT in 0.1 ml DMSO or 0.1 ml DMSO alone. Litter size averaged 8 pups per litter and sacrifice was at 102 to 138 days of age.

[2] Body weights are given in grams in parentheses under each heading.

Table 9 (Cont.)

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[3] All data are given as mean weights or mean % of body weight ± 1 standard deviation; numbers in parentheses after the treatment designation are the numbers of animals examined; comparisons are all versus the appropriate control groups and are made by unpaired Student's t-tests, * $p < 0.05$, ** $p < 0.025$.

1.04 ± 0.32 and 0.86 ± 0.39 ug B-640/ml for control and DDT-treated groups, respectively. Nor did a different group of pituitaries (Section V, this Chapter), taken from animals treated via their dams, demonstrate weights differing from those found in directly injected animals for either control or DDT-exposed groups. Possible explanations of the present results include variability in litter sizes during the experiment, possible problems with malnutrition due to the presence of primiparous dams and/or inexperience or inconsistency in dissection and handling of the pituitary. Whatever the cause, the later determinations, on intact rats, similarly dosed in litters of 6 and 12 pups (Section V, this Chapter), gave values for normalized pituitary weights of 2.59 ± 0.24 for control animals (21 animals) and 2.69 ± 0.25 for DDT-treated animals (20 animals); both values agree with those found in directly injected groups. Similarly, a later determination of the normalized hypophysial weights of neonatally castrated, suckled rats raised in 8 pup litters showed values of 3.74 ± 0.30 and 3.37 ± 0.14 mg/100 g body weight for control (2 animals) and DDT-treated (3 animals) groups, respectively; values again in accord with those determined for directly injected animals.

The above results yielded no clue as to any possible effect of o,p'-DDT on the male rat. They again, as was the case with directly injected rats, implied the need for the use of more sensitive measurements. To that end, and with the data of Rybakova (29,29) and Gellert et al. (53, 54,150,151) in mind, several experiments were done to probe aspects of pituitary release of LH.

III. Periodicity of LH Release in Adult Rats Neonatally Exposed to DDT:

Adult castration superimposed on neonatal DDT-treatment was shown to expose a measurable difference between the responses of sesame oil and DDT-treated LH control systems (Chapter 3, Section III). Ether had analogously been shown to alter the 24-hour periodicity of serum LH levels in untreated animals (Chapter 2, Section IV.D.2.) (183-186). The following experiment, though originally performed to measure the direct effect of o,p'-DDT exposure on the 24-hour periodicity of serum LH in both intact and neonatally castrated rats, is best discussed in light of the effect of superimposing a second stressor, ether, on what may be an LH control system which has already been perturbed by neonatal exposure to o,p'-DDT. Differences in ether response throughout the day are, in fact, what was measured.

Two groups of rats were subdivided into 2 subgroups each, 2 of which were treated with o,p'-DDT via their dams (DDT-treated) and 2 of which were suckled by vehicle injected dams (Controls). One subgroup under each treatment was castrated on the day of birth. The pups were reared in 8 pup litters and were used for the experiment at 76-89 days of age. After weaning, a pair of subgroups, one DDT-treated and one control, were each split into 3 sets of 2-5 animals each, i.e., 3 sets of 2-5 control animals were formed and 3 sets of 2-5 DDT-treated animals were formed. Beginning at 09.00 or 11.00 (EST) individual animals from a pair of the sets (one DDT-treated set and one control set) were alternately bled under light ether anesthesia (TVBE). Animals were returned to their cages and to the normal light/dark cycle when fully conscious after bleeding; total elapsed time between removal of an animal from the cage to its return was about 6-8 minutes. The serum was separated from the collected blood and stored at -20°C until analysis by the RIA procedure of Niswender et al. (169). The bleeding procedure was repeated at 2 hour intervals, using the sets of animals pairwise and in rotation so each animal was bled once every 6 hours, i.e., a total of 4 times over the course of the test period.

Means for each treatment group and each time of day were calculated from the results obtained from the RIA. The means were plotted versus time of day and visually examined for the existence of any discernable circadian rhythm(s). Corresponding means for DDT-treated and control rats were tested for difference by Student's t-tests. Both the DDT-treated and the control group results for intact animals were also divided into halves, before 20.00 and after 20.00 (EST), and examined in a 2-way ANOVA for the effects of both time, day and night, and exposure to DDT.

The LH patterns obtained for both neonatally castrated and intact rats are shown in Figure 31. No significant differences between DDT-treated and control groups were found at any single time of day for either neonatally castrated or intact animals. No rhythm was shown in either treatment group of castrated rats. The levels of LH seen in castrates were similar to those reported by Lawton and Smith (186). The intact animals generated a picture similar to that seen in Figure 8 which describes intact, normal, adult males exposed to ether. In intact males the LH values found during the day were more variable and higher ($p < 0.005$) than those obtained at night. A superficial rise near 24.00 (EST) may reflect the LH peak which coincides with peak activity (see Chapter 2, Section IV.D.2. and Figure 8), however, the values are too near the reliable limit of the assay to distinguish that point from any others between 18.00 and 06.00 (EST). Though there was no significant difference seen in LH values between the entire set of results from the control and DDT-treated intact groups ($0.05 < p < 0.10$), the combined values for the results between 15.00 and 19.00 (EST) did differ for the two groups ($t_{15} = 2.4625$, $p < 0.05$), the controls being higher than the test group.

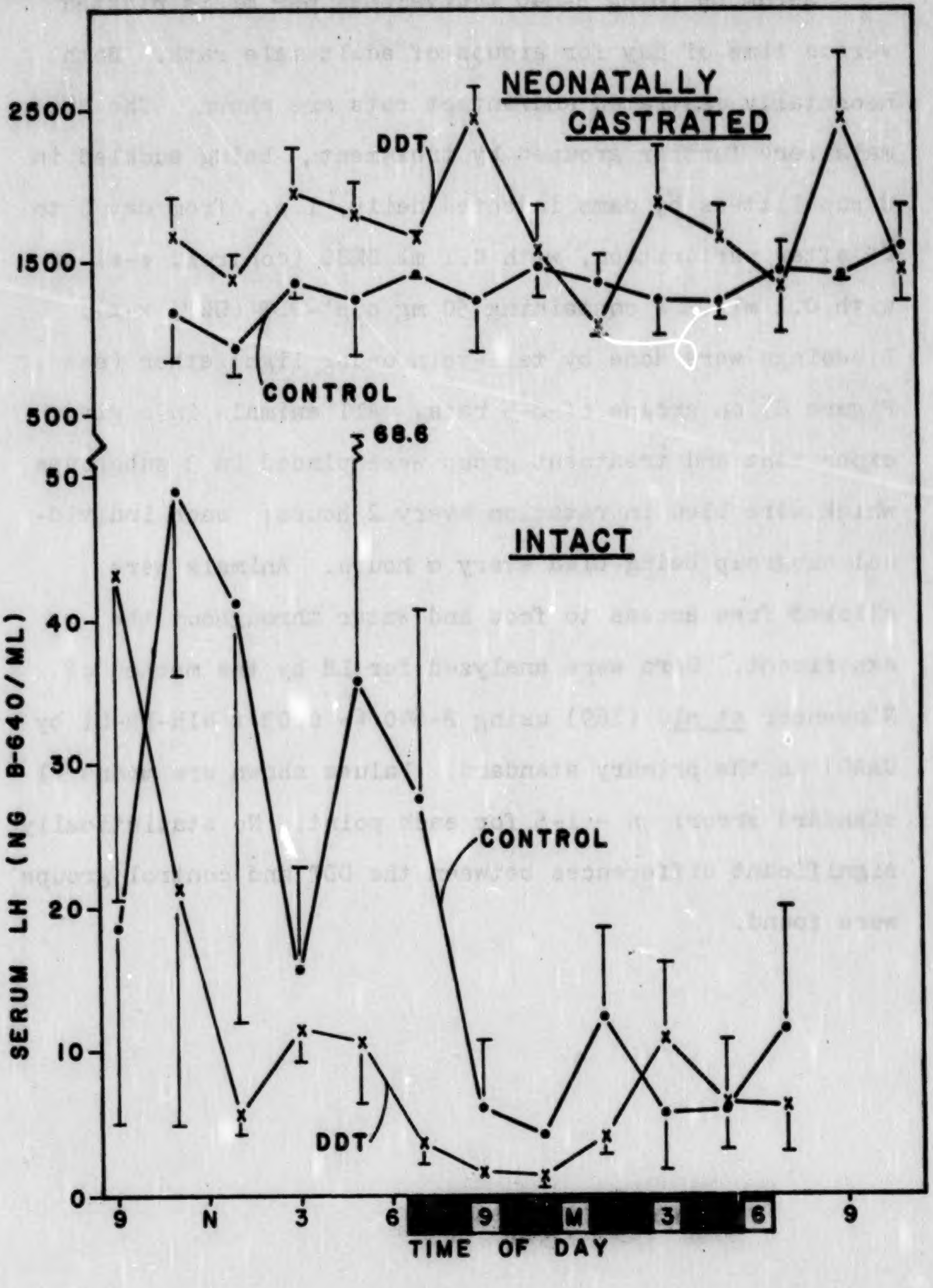
The possibility that the castrates demonstrated a larger ether effect than the intact animals was not supported by the similarity of the present results to the values of serum LH in long-term castrates bled by

Figure 31. 24-Hour Periodicity of Serum LH in Adult Male Rats Treated Neonatally Via Their Dams

Serum LH in ng B-640 equivalents per ml is plotted versus time of day for groups of adult male rats. Both neonatally castrated and intact rats are shown. The animals were further grouped by treatment, being suckled in 8 pup litters by dams injected daily, i.p., from day 0 to 25 after parturition, with 0.1 ml DMSO (control, ●-●) or with 0.1 ml DMSO containing 50 mg o,p'-DDT (DDT, x-x). Bleedings were done by tail-vein under light ether (see Figure 8) on groups of 2-5 rats. All animals in a given experiment and treatment group were placed in 3 subgroups which were bled in rotation every 2 hours; each individual subgroup being bled every 6 hours. Animals were allowed free access to food and water throughout the experiment. Sera were analyzed for LH by the method of Niswender et al. (169) using B-640 (= 0.03 x NIH-LH-S1 by OAAD) as the primary standard. Values shown are means ±1 standard error; n = 1-5 for each point. No statistically significant differences between the DDT and control groups were found.

FIGURE 31

24-HOUR PERIODICITY OF SERUM LH IN ADULT MALE RATS TREATED NEONATALLY VIA THEIR DAMS



decapitation as published by Yamamoto et al. (180). The possibility that DDT-treated rats showed an ether response which compensated for a depression in LH caused by o,p'-DDT cannot be ruled out. However, the observation that a response to ether did not eliminate the difference between DDT-treated and control animals castrated as adults (Chapter 3, Section III) argues against such compensation. The present finding that no difference was seen due to o,p'-DDT exposure when a difference did occur after adult castration in directly injected animals may have been due either to the longer period which had elapsed after castration - serum LH continued to rise for at least a month after castration (180) - or to the difference in the absorbed dosage (pups treated via the dam absorbed less than 0.5 mg over a period of 25 days while injected pups received 10 mg over 5 days).

The conclusion reached from the experimental results was that neonatal exposure to o,p'-DDT does not significantly alter the 24-hour periodicity of the response of serum LH to tail-vein bleeding under ether in either neonatally castrated or intact rats. There does appear, however, to be a tendency toward lower values in intact DDT-treated animals at least during the late afternoon.

IV. Serum LH Response to Adult Castration:

Since no significant alteration of the circadian pattern of LH response to ether was generated by neonatal DDT exposure in the preceding experiment, an experiment was undertaken to measure the effect of neonatal DDT-exposure on the LH response to adult castration. The experiment was later repeated in directly injected animals (the experiment discussed in Chapter 3, Section III). As was the case in the other experiments dealing with LH release the empirical bases for the experiment were the observations of Rybakova (28,29), showing increased pituitary LH levels in male and female rats chronically fed technical DDT, and the observations of Gellert et al. (54), showing the blunting of the response to adult castration in female rats neonatally injected with 0.001-1.0 mg of o,p'-DDT.

The experiment was initiated with two litters of 8 neonatal pups each. One litter received o,p'-DDT via its treated dam (DDT-treated) while the dam of the other litter received vehicle only (control). Attrition due to undetermined causes during development and prior to the beginning of the experiment on day 57 of age, limited the number of animals to 3 in the control group and 4 in the DDT-treated group.

To establish a response baseline serum samples were taken between 15.00 and 18.00 (EST) by TVBE on days 57, 58 and 59; the sera were stored for later assay. On day 60 the animals were castrated but no blood samples were taken. On days 1, 3, 6 and 10 following castration serum was obtained from all the rats by TVBE at 15.00-18.00 (EST). Two rats succumbed to ether and a preexisting respiratory infection leaving 2 control rats and 3 test animals. An injection of testosterone (0.75 mg/kg) was given 6 hours prior to bleeding on day 14 to ascertain if the gonadal-hypothalamo-hypophysial feedback loop was functioning differently in the DDT-treated and control animals (170). Finally, on day 15 postcastration, the animals were anesthesized with ether and decapitated; trunk-blood was collected to determine if LH had rebounded normally from the testosterone injection.

Sera were analyzed by RIA using the procedure of Niswender et al. (169). Means were calculated for each experimental day and each treatment; a plot of these results is presented as Figure 32. Two-way ANOVA was also done on the values obtained between the time of castration and the time of testosterone administration, i.e., on the results from days 1-10.

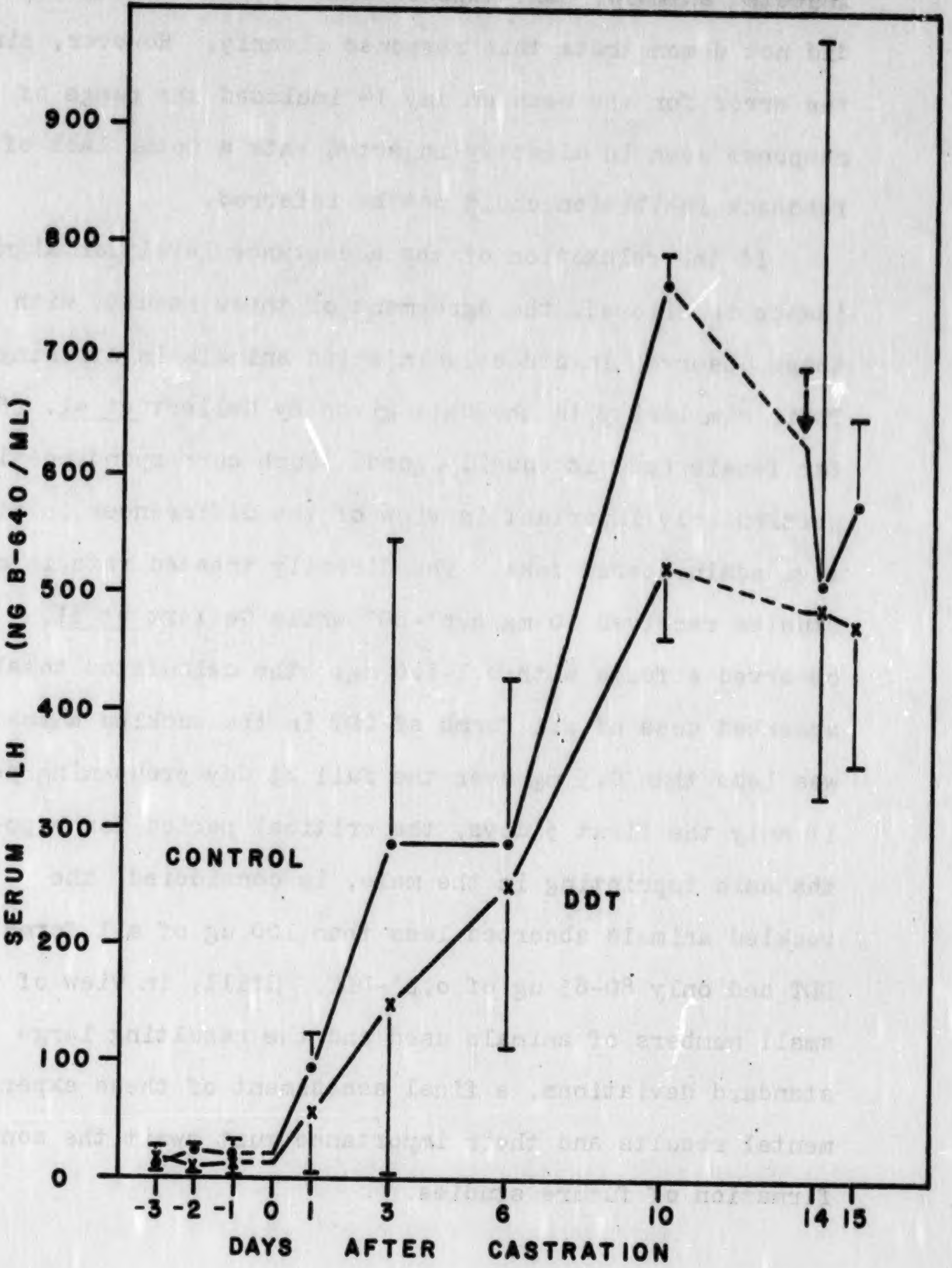
Due to the small number of animals involved the standard deviations were quite large. At the 5% significance level no difference was apparent between the control and DDT groups. If the acceptance level for significance was relaxed to 10%, however, the control showed significantly higher values than the DDT-treated animals during the period of 1-10 days postcastration; values for DDT-treated animals were 15-20% lower than those for the controls. The measured LH values for the period following day 6 were quite similar for both control and DDT-treated groups to those encountered in the better balanced experiment done on directly injected animals. Controls

Figure 32. Response of Serum LH to Adult Castration in
Male Rats Treated Via Their Dams

Serum LH as ng B-640 (= 0.03 x NIH-LH-S1 by OAAD) equivalents per ml is plotted versus time after adult castration for two groups of male rats. The control rats (●-●) were suckled prior to weaning in a litter of 8 pups by a dam which received daily i.p. injections of 0.1 ml DMSO while the DDT-treated rats (x-x) were reared in a litter of similar size by a dam which received daily i.p. injections of 0.1 ml DMSO containing 50 mg o,p'-DDT. The animals were reared with free access to food and water and were castrated at 60 days of age. Blood samples were taken from the same animals throughout the experiment by TVBE at 15.00-18.00 (EST) on the days indicated. On day 14 an injection of 0.75 mg/kg testosterone (T) was given s.c. in sesame oil 6 hours prior to bleeding. Sera were assayed by the method of Niswender et al. (169). Values shown are means ±1 standard deviation for groups of either 2 control or 3 DDT animals. Two-way ANOVA of the results between castration and testosterone injection (days 1-10) yielded a p value between 5 and 10% for the difference between control and DDT-treated groups.

FIGURE 32

RESPONSE OF SERUM LH TO ADULT CASTRATION IN MALE RATS TREATED VIA THEIR DAMS



demonstrated a response to testosterone administration which appeared similar to that observed in directly injected animals. DDT-exposed animals, on the other hand, did not demonstrate this response clearly. However, since the error for the mean on day 14 included the range of response seen in directly injected rats a total lack of feedback inhibition could not be inferred.

If the relaxation of the acceptance level for significance is allowed, the agreement of these results with those observed in directly injected animals is striking. Their similarity to the data given by Gellert _et al_. (54) for female rats is equally good. Such correspondence is particularly important in view of the differences in effective administered dose. The directly treated rats in my studies received 10 mg o,p'-DDT while Gellert _et al_. observed effects with 0.1-1.0 mg; the calculated total absorbed dose of all forms of DDT in the suckled animals was less than 0.5 mg over the full 25 day preweaning period. If only the first 5 days, the critical period for hypothalamic imprinting in the male, is considered, the suckled animals absorbed less than 100 ug of all forms of DDT and only 80-85 ug of o,p'-DDT. Still, in view of the small numbers of animals used and the resulting large standard deviations, a final assessment of these experimental results and their importance must await the confirmation of future studies.

V. Serum LH Response to Exogenous LHRH:

Studies by Rybakova (28,29) indicated a change in the pituitary content of LH in rats chronically fed low levels of technical DDT. Other studies by Arai (211) had shown changes in pituitary stores of LH and hypothalamic stores of LHRH in response to neonatal estrogen treatment. Several of the experiments already discussed also implied a change in the function of the overall feedback system controlling gonadal steroidogenesis. On the basis of these observations, and in order to begin answering the question of the locus of action of neonatal o,p'-DDT, a measurement of the response of this feedback system to exogenously administered LHRH was undertakon.

The design of the experiment included the use of two different litter sizes and two periods of injection of the dam in order to probe the results of different absorbed dosages. A 3-level factorial design was generated for 8 litters; a control and a DDT-treated group were constructed in each of 2 litter sizes (6 and 12 pups per litter) and at each of 2 durations of injection of the dam (5 and 25 days). Growth was monitored by regularly checking body weights. Beginning at 149-154 days of age 4-6 animals from each litter were subjected to a test of their serum LH response to injection of synthetic LHRH. Rats derived from dams injected for 5 days were tested separately from those reared by dams injected for 25 days.

Treatment Groups

	Treatment of the Dam	
Litter Size	Injected Chemical	Number of Injections
6	DMSO	5
6	DMSO	25
6	o,p'-DDT	5
6	o,p'-DDT	25
12	DMSO	5
12	DMSO	25
12	o,p'-DDT	5
12	o,p'-DDT	25

On the third day of each test[1] simple TVBE was done at 17.00-19.00 (EST) to generate a basal level of LH for reference. The animals from the 6-pup litters in both the control and DDT-exposed groups were bled in rotation (to prevent temporal bias) followed by those from the 12-pup litters. On the fourth day the same rotation was followed except that, before bleeding each group, each animal received a 1.0 ug i.p. injection of synthetic LHRH in 0.1 ml of saline. The bleedings were begun 20 minutes after injection and were completed on each group less than 60 minutes after injection. The same procedure, including LHRH injection, was repeated on the fifth day of the test.

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[1] The test results for the present experiment were plotted beginning on day 3 to allow comparison of LH levels to the range of LH values seen in a group of rats which were neonatally injected with sesame oil and bled as adults on 8 consecutive days during which they were injected with saline on days 3-6.

On the sixth day no bleeding or injections were performed. On the seventh day of the test, animals were weighed, stunned, decapitated, bled and autopsied. Weights and organ weights were measured as previously described and assays for LH were done on both sera and pituitary extracts. Results for the LH assays were plotted versus day of the test and analyzed for differences by a 3-way ANOVA as well as by individual t-tests between the means of each DDT-treated group and its paired control (paired both in litter size and length of dam injection) on each day of the experiment. Student's t-tests were likewise used to compare values for body weights, etc., from each paired control and DDT-exposed litter.

Growth for litter sizes, of 6 and 12 pups diverged as expected (163,216) between 5 and 10 days of age; o,p'-DDT injection into the dams for either 5 or 25 days did not alter weight changes. Pups from 6-pup litters grew slightly more rapidly than those from 8-pup litters while pups from 12-pup litters grew somewhat more slowly than those from 8-pup litters. Final body weights for rats from 6-pup litters were fully 10% above those of rats from 12-pup litters (significant at $p < 0.001$). Normalized organ weights, however, did not differ because of difference in litter size, DDT treatment or length of DDT-injection into the dam.

A representative plot of the experimental results obtained for serum LH is shown in Figure 33 - values

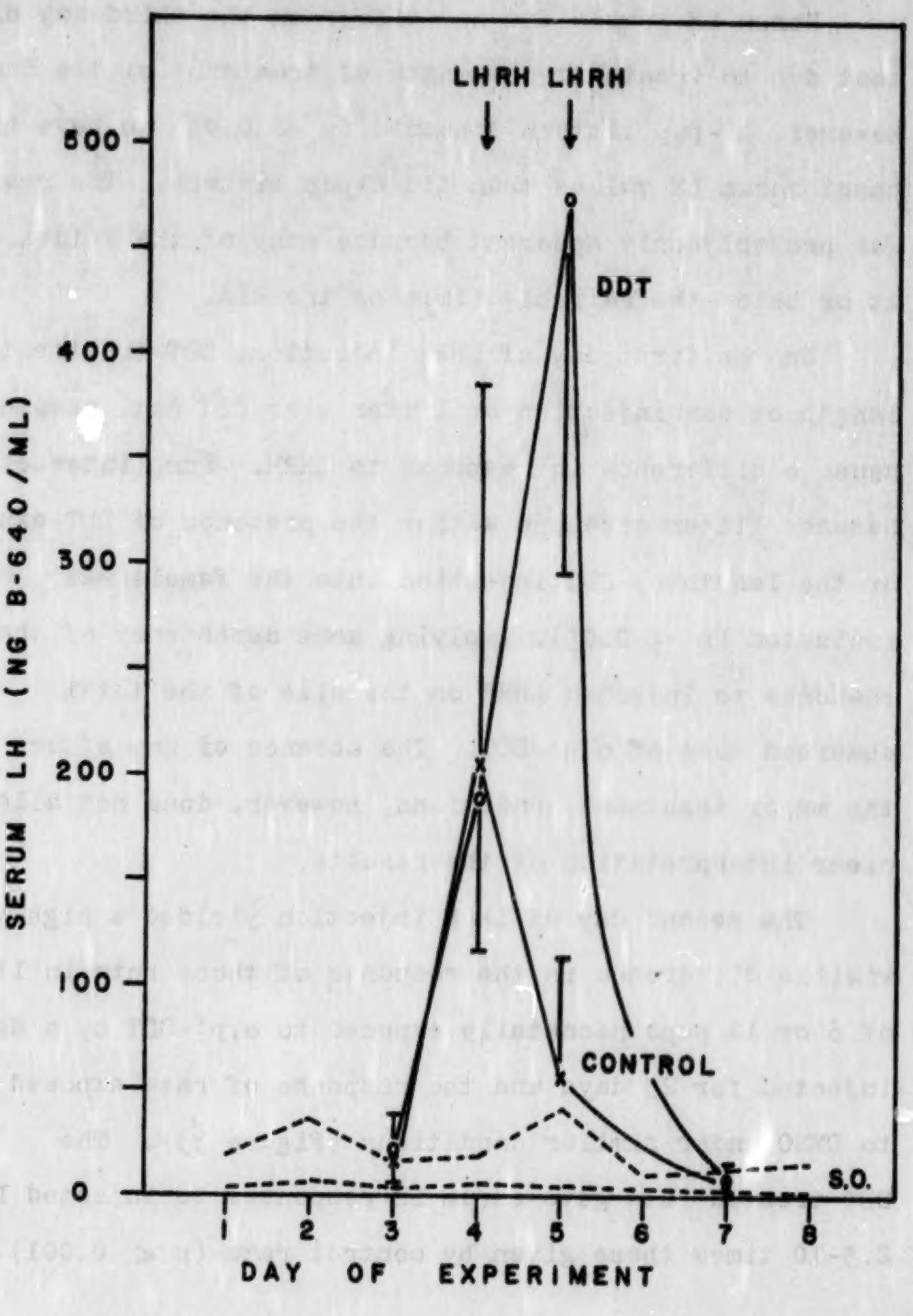
Figure 33. Response of Serum LH to Injection of Large Doses of LHRH in Adult Male Rats Treated Via Their Dams

The results of a test of the pituitary response to repeated injection of LHRH in vivo is shown. Serum LH is plotted versus time for 2 groups of rats raised in 12-pup litters; Controls (x-x) which were suckled prior to weaning by a dam which received daily i.p. injections of 0.1 ml DMSO , and DDT treated animals (o-o) which suckled a dam which received daily i.p. injections of 0.1 ml DMSO containing 50 mg o,p'-DDT. The 149-154 day old animals, which had free access to food and water throughout the experiment, were bled at 17.00-19.00 (EST) on the days shown by TVBE. Simple bleedings were performed on days 3 and 7 but 20-60 minutes (unbiased between groups) prior to the bleedings on days 4 and 5 the animals were injected i.p. with 1.0 ug of synthetic LHRH in physiological saline. Sera were assayed by the method of Niswender et al. (169) using B-640 (= 0.03 x NIH-LH-S1) as standard. Values shown are the means ±1 standard deviation for groups of 4-6 rats. A Student's t-test for day 5 of the experiment shows a large ($p < 0.005$) dependence on DDT treatment for the observed response to the second daily injection of LHRH.

Simple bleedings were also done on a group of male rats which received neonatal injections of sesame oil (S.O.); the limits of the range of their LH levels (===) is shown for comparison with the LHRH treated animals.

FIGURE 33

RESPONSE OF SERUM LH TO INJECTION OF LARGE DOSES OF LHRH IN ADULT MALE RATS TREATED VIA THEIR DAMS



obtained for rats reared in 12-pup litters and suckled by dams injected for 25 days. Plots for the other litter sizes and for the other length of dam injection have not been included.

Serum LH levels did not differ on the third day of the test due to treatment or length of treatment of the dam. However, 12-pup litters appeared ($p < 0.05$) to have higher basal serum LH values than did 6-pup litters. The result was probably only apparent because many of the values were at or below the reliable limit of the RIA.

On the first day of LHRH injection, DDT-treatment, length of dam injection or litter size did not, themselves, cause a difference in response to LHRH. Some interaction between litter size and either the presence of DDT-exposure or the length of DDT injection into the female was indicated ($p < 0.05$), implying some dependency of the response to injected LHRH on the size of the total absorbed dose of o,p'-DDT. The absence of any effect of the major treatment conditions, however, does not allow clear interpretation of the results.

The second day of LHRH injection yielded a highly visible difference in the response of those rats in litters of 6 or 12 pups neonatally exposed to o,p'-DDT by a dam injected for 25 days and the response of rats exposed only to DMSO under similar conditions (Figure 33). The DDT-treated rats gave serum LH responses to injected LHRH 2.5-10 times those given by control rats ($p < 0.001$).

Interaction between treatment and both length of treatment alone ($p < 0.005$) and length of treatment and litter size ($p < 0.05$) imply that total dosage of o,p'-DDT may have been the deciding factor in the observed results. Rats neonatally suckled by females injected for only 5 days did not differ from their controls.

No differences among LH values from any of the groups or treatments was evident in the sera collected at the termination of the experiment. The LH values of the pituitary extracts (not presented) implied a dependence of pituitary LH content on the length of treatment of the dam ($p < 0.005$), i.e., on the number of injections given the dam or, possibly, on slightly different amounts of injected LHRH for the animals in one half of the experiment (reared by dams injected for 25 days) versus those in the other half (reared by dams injected for 5 days).

Interpretation of the results of this experiment is definitely complex. First of all, unintentional experimental differences between the animals derived from dams injected for 25 days and those derived from dams injected for 5 days may have arisen because the former group was tested for LHRH response 5-10 days before the latter. This may have generated artifactual differences which would be reflected in significance of the experimental factor associated with the length of the injection period for the dam regardless of the presence or absence of DDT-treatment. If, for example, LHRH degraded measurably during storage in solution, the responses to LHRH for the animals reared by dams injected for 5 days and injected with stored LHRH would be less marked than those in animals reared by dams injected for 25 days and injected with freshly diluted LHRH. Such a difference would explain the overall lower serum and pituitary LH values seen in the second group tested and would also explain the absence of a measurable potentiation of the response to the second LHRH injection in rats suckled by dams injected for only 5 days. If, on the other hand, no such problems occurred, the difference in the results for the two dam injection periods might be explained on the basis of lower doses having been received by those animals suckled by dams injected for only 5 days. Or they might be explained on the basis of nutritional changes, which were not reflected in body weight and which potentiated the effect(s) of DDT, having occurred in rats suckled by dams injected for 25 days.

Whatever the cause of the difference in outcome related to the 2 different treatment periods, the experiment dealing with animals suckled by dams injected for 25 days with o,p'-DDT did demonstrate a marked increase of the serum LH response to the second injection of LHRH. In rats from both litter sizes the potentiation in DDT-exposed rats was coupled with a decrease in response in control animals. This may indicate that the two groups differ in either their rates of LHRH degradation or their rate of

LH synthesis. Though the amount of LHRH injected was large, calculation of the effective concentration following injection, based on the half-life of 6.5 minutes given by Schally et al. (9), indicated that the injected dose would be cleared in less than 5.3 hours. Even slowing this down 2-3 fold would not explain the results on the basis of residual LHRH. Potentiation based on the priming effect of closely spaced injections of LHRH (172) would similarly not be favored due to the length of time between the doses of LHRH.

The remaining possibilities for explaining the differences seen between the control and DDT exposed groups may involve altered sensitivities of target organs in the hypothalamo-hypophysial-gonadal system to circulating hormones as would be caused by neonatal imprinting or they may involve the continued presence of residual DDT which interferes with the feedback suppression of pituitary LH release and/or synthesis. The second mechanism is highly unlikely since measurements of DDT residues in 120 day old females injected neonatally with 3 mg of o,p'-DDT demonstrated no more residues than control rats (53). Since the animals in the present experiment were even older male animals, which should have metabolized DDT even faster than females (105,107), residues should not act directly during the period used to measure the responses to LHRH. The alternative mechanism, i.e., imprinted sensitivity changes which are expressed in the presence of high levels of circulating LHRH, is in concert with my earlier findings of probable alterations in the gonadal steroidogenic control loop. The opposite direction of the changes in serum LH seen in the DDT-treated rats in the response to adult castration and the response to repeated LHRH injections may be manifestations of different DDT effects or merely the expression of a different facet of the same effect.

The smaller response of serum LH observed with the second injection of LHRH, relative to the first, in control animals may be produced by a depletion of releasable pituitary LH stores. Initially, this would be due to the presence of massive amounts of LHRH. Subsequently it might be due to direct suppression of LH synthesis within the depleted pituitary by the large quantities of testosterone generated as a result of the LH release brought about by the first injection of LHRH. The precise mechanism is not clear at this time.

In summary, treatment of neonatal animals with o,p'-DDT via an injected dam appears quite effective. Nearly all of the o,p'-DDT which enters the gut in the milk was absorbed by suckling pups. Such treatment was not effective in altering body or organ weights, as might have been anticipated on the basis of both the calculated absorbed dose and the previous findings in the present studies. DDT-treatment via the dam was not effective in altering overall serum LH periodicity. It was, however, effective in modifying the serum LH responses to adult castration and to repeated large injections of LHRH.

CHAPTER 5

DISCUSSION OF ANIMAL STUDIES

This study began, experimentally, as an attempt to measure direct interaction between the metabolism of o,p'-DDT and the androgenic steroids in the testes of adult male rats chronically exposed to o,p'-DDT, i.e., it began as a study of interactions _in vitro_. During the course of initial experiments, however, technical problems due to a variety of causes limited the results obtained to a few very imprecise observations which indicated little or no effect of DDT exposure. At that juncture (1971) a growing awareness of the literature made it apparent that very few effects of DDT or its analogs had been observed - or tested for - _in vivo_, much less _in vitro_ - in the steroidogenic tissues of any mammalian species; the effect of o,p'-DDD on the adult adrenal in several species was the only real exception. Since the available literature indicated only that o,p'-DDT was a weak estrogen (22, 24,146,148,152) the best approach to the problem appeared to be to conduct a brief but very broad study _in vivo_ which would indicate which portions of the endocrine system would be most profitably studied by subsequent studies run _in vitro_. Body and organ weights seemed to be the most appropriate measurements for such a screening experiment since they should indicate changes which might occur in the the adrenals, the gonads, the liver and the hypothalamic-pituitary complex. Before the study was begun, however, a report by Heinrichs et al. (53) became available. It indicated that some marked changes occurred in the steroidogenic system of the female rat following neonatal injection with moderate doses of o,p'-DDT. The approach of using neonatal injection appeared profitable both because it yielded quite demonstrable changes and because it allowed the use of a large body of literature on neonatal steroid imprinting in the rat (57-63,69-71) as a background for comparisons. After several short experiments using the neonatally injected male had failed to demonstrate any changes in adult organ weights, it was decided that a longer study incorporating several ages, several dose levels and examinations of organ histologies, serum corticosterone and serum LH might prove more illuminating. This study, described in Chapter 3, Section II, did yield an indication that development of adrenal morphology, and possibly function, was effected by neonatal injection with a range of concentrations of o,p'-DDT. The experiment, however, also indicated that if any changes in parameters dealing directly with gonadal steroidogenesis had occurred they were rather subtle. Subsequent experiments (Chapter 3, Section III) based on the work of Rybakova (28,29) and Gellert et al. (54) concentrated on measurements of serum LH under conditions which stressed part of the hypothalamo-hypophysial-gonadal axis. They were designed to determine "reserve" capacity, i.e., the capacity to respond to stress. Adult castration was found in such experiments to generate a picture of altered "reserve" or response capacity in male rats neonatally injected with o,p'-DDT.

While these experiments on injected rats were being conducted a parallel series was initiated using the natural source of environmental dietary contaminants for the pup, i.e., the mother's milk, as the vehicle for neonatal exposure to o,p'-DDT. Again, as with directly treated animals, the results indicated that some changes in the capacity of the gonad-brain-pituitary axis had occurred due to DDT exposure but that the changes were exposed only by adult castration or exogenous LHRH. However, the changes occurred at moderately low levels of exposure; 100 ug of DDT in a 5 day old, 10 g pup is a body burden of 10 ppm which is near the levels found in some environmental exposures. Taken together the results from the two sets of experiments, in directly injected and suckled rats, indicated that at least two loci of o,p'-DDT effects were demonstrable within the realm of the steroidogenic systems: 1) the immature adrenal cortex, and 2) the hypothalamic-pituitary complex.

I. The Change in the Immature Adrenal Cortex:

The adrenal has been implicated in the onset of puberty in the female rat (98) and in the maturation of the testicular response to hCG (78,79). If the process of maturation of the adrenal is interfered with it is conceivable that the maturation of other tissues influenced by the adrenal will be affected. The coordination of the rise and fall of several hormone levels can have marked influence on the normal maturation of tissues, e.g., only the proper coordination of LH, FSH and E_2 will allow a developing follicle within the ovary to develop to the stage of ovulation; other conditions result in atresia (89). Because of this, either adrenal degeneration, or a precocious adrenal development, as was found in these studies, may result in pathologic states which could effect the development of any of a myriad of other tissues which are known to be effected by the function of the adrenal cortex (206).

If precocious development is viewed as accelerated aging, then the precocious development of the adrenal cortex seen in animals treated neonatally with estradiol or o,p'-DDT may result in eventual degeneration similar to that seen after treatment of the adult adrenal of several species with o,p'-DDD. Since the adrenals of o,p'-DDT-treated rats did not exhibit the specific type of involuted morphology seen in other species treated with o,p'-DDD (37-49) the DDT effects seen may not be the result of this metabolite. Furthermore, since the adult rat exhibits a refractoriness to the action of o,p'-DDD it is improbable, though not impossible, that o,p'-DDD also would be effective in the neonate.

A more likely explanation for the results is that the estrogenicity of o,p'-DDT may be the cause of its action on the adrenal. The adrenal is known to be positively effected both morphologically and functionally by the presence of circulating estrogen (4). This is true functionally from at least 25 days of age onward and morphologically (normallized weight) from 50 days onward (see Chapter 3, Section II). Since steroids influence tissues by way of intracellular mechanisms subsequent to binding a steroid receptor, it may be assumed that o,p'-DDT may function by binding to estradiol receptors. DDT has been shown to bind to uterine estradiol receptors with affinities of the order of 1-10 x 10^{-6} M (K_d) - affinity for estradiol is 4 x 10^{-10} M (26). It has also been shown to bind to hepatic P-450 (221) and adrenal P-450 (222,223) in such a manner that the substrate difference spectra of P-450-steroid and P-450-DDT are similar (Type I). Furthermore, the estimate of estrogenic potency found (Chapter 3, Section II.C.) for intermediate dosages of o,p'-DDT on the basis of corticosterone secretion agrees with that found using a totally different assay of estrogen potency, the rat uterine-glycogen bioassay (147).

Still, there is the element of the time lag between injection and the observation of an effect which must be explained before an adequate hypothesis based on estrogenicity is possible. The potential for the sesame oil vehicle to act as a depot has already been discussed in regard to the steroids; the same arguments apply to o,p'-DDT. Another possible mechanism of estradiol and o,p'-DDT action would be via a peripheral imprinting of the adrenal cortex analogous to those encountered in the gonads and/or the liver. Finally, the adrenal precosity found in animals neonatally injected with estradiol or o,p'-DDT may be an indirect effect of hepatic microsomal induction. In this instance an early compensatory hypertrophy of the neonatal adrenal cortex would take place to maintain serum corticosteroid levels and would not have fully regressed by 25 days of age. This explanation is, however, unsatisfactory on two bases: 1) estradiol in adult rats inhibits microsomal metabolism and induction (5), and 2) the serum levels of corticosterone are higher than normal. Still, unless the levels of free corticosterone in serum are measured rather than total corticosterone (which includes corticosterone bound to corticosteroid-binding-globulin (CBG)) the actual effective level of the hormone in serum is unknown and the second argument is weakened.

Obviously, a great deal of research remains to be done to clarify the meaning and significance of the observed early development of the adrenal cortex in treated animals. If the present studies were to be carried forward a repeat of the first 20-30 days of the direct injection study using several doses of o,p'-DDT dissolved in DMSO or ethanol and similar doses dissolved in sesame oil could be used to verify the initial observations while eliminating the question of long-term, depot-injection effects. Such studies would also provide tissues for some of the following measurements:

1. Measurements of cholesterol conversion into glucocorticoids in quartered adrenals in the presence or absence of ACTH and/or estradiol or o,p'-DDT would determine the level of functionality of the adrenal at the time the altered histological appearance was in evidence and would also test for the altered adrenal's sensitivity to ACTH, estradiol and o,p'-DDT.

2. Measurements of serum corticosterone and CBG would establish the amount of free serum corticosterone and could be used to help differentiate direct effects on the adrenal from those mediated by the liver.

3. Measurements of hepatic microsomal breakdown of corticosteroids would also differentiate direct effects on the adrenal from those mediated by the liver.

4. Measurements of serum ACTH would help to determine whether the current observation is due to the continued presence of estradiol or o,p'-DDT or to an imprinted change in pituitary output of ACTH or hypothalamic production and release of CRH.

Other studies involving surgical manipulations, e.g., hypophysectomy, of the dosed animals are, of course, possible. However, their interpretation would probably be less precise than the more direct measurements listed above.

II. The Change in the Hypothalamic-Hypophysial Complex:

Because LH (and FSH) are involved in controlling steroidogenesis and spermatogenesis the observed changes in the hypothalamic-pituitary axis probably have the potential for a more direct effect on reproductive capacity and/or development than those described for the adrenal. However, the apparent normality of the development of the DDT-treated animals argues against such an effect. On the other hand, it is doubtful whether any such change would manifest itself in altered reproductive performance in laboratory rats for whom the stresses of obtaining and maintaining a mate are in most situations considerably less than in animals in the wild state. Therefore, situations in which a somewhat abnormal LH response capacity might play a role in depressing reproductive success such as courtship or repulsion of rivals may not occur in tests of reproductive capacity with such laboratory rats.

The subjective appearance of at least transient oligospermia in DDT-injected males could result directly in reduced reproductive success. The observations of Krause et al. (208) might be used at this point to support the argument for oligospermia and reduced fecundity, however, the quantities of DDT which were administered in that study could well have caused hepatic induction and testosterone breakdown long after their injection. For that reason that study is potentially misleading and cannot be used as clear support for an argument favoring a hypothalamic or pituitary alteration resulting in impaired spermatogenesis in DDT-treated rats.

Even if the observed changes in LH response capacity does not have any effect on reproductive success it is still of interest from the viewpoint of understanding the mechanisms controlling LH synthesis and release, i.e., endocrine control. To my knowledge it is the first observation in the male rat of long term effects caused by a chemical agent on hypothalamic-pituitary responses to adult castration or LHRH injection. Therefore, it leads to some interesting speculations concerning the mechanism of the response and provides a base for future experimentation. The apparent differences in the directions of the two effects (LHRH-response and adult castration response) may be based on preservation of the cyclic center of the hypothalamus in DDT-treated male rats but not in the controls (Chapter 1, Section II). Such a preservation might occur by occupation of the center's estrogen receptors by the very weak estrogen, o,p'-DDT, during the critical phase of hypothalamic development. After the critical imprinting period had passed and the dosage had been discontinued the o,p'-DDT (or the actual active metabolite) would be gradually eliminated by catabolism. Since endogenous testosterone would thus be unable to act neonatally, the cyclic center would remain intact. Adult castration, which removes the source of negative feedback onto the tonic center (57-61), would not reveal the presence of such an intact cyclic center. But large doses of exogenous LHRH, LH, testosterone or, possibly, estrogen might stimulate an LH surge of the type seen in the cycling female rat near ovulation. These agents could cause the appearance of an LH response superimposed on an LH surge on the second day of LHRH injection in DDT rats which would not be possible in control rats. This would be the case because the endogenous source(s) of LHRH, which would be necessary for replenishment of pituitary LH stores after the first injection with LHRH, would be suppressed by the testosterone produced as a result of the first LHRH injection in the control but not in the DDT-treated rats. This mechanism would not explain the lower plateau of the DDT-treated rats after the adult castration unless some change in the tonic center had also occurred during its period of exposure to o,p'-DDT. Still, simultaneous changes in both cyclic and tonic centers have been described for neonatal treatment with androgens and estrogens (61) and thus would not be implausible for o,p'-DDT.

A second more inclusive explanation would involve either a continued presence of o,p'-DDT which could bind to hypothalamic steroid (estrogen?) receptors or an imprinting which caused a modification in the number or affinity of the testosterone (estrogen?) sensitive receptors within the brain. Either of these conditions would bring about a state in which a fraction of the total steroid receptors were, in effect, inactive or occupied by a weak steroid analog. This fraction would be unavailable to act in totally suppressing LHRH release but would constantly function at a low level to suppress unstressed LH levels. This situation would result in an inability of testosterone, generated perhaps by a burst of LH released in response to a single LHRH injection, to fully suppress LHRH release and therefore to prevent stimulation of the replenishment of releasable LH within the pituitary. It would also result in an effectively slower fall of serum testosterone relative to receptor concentration after castration in DDT-treated as opposed to control animals. This, in turn, would cause a slower rise in serum LH following castration. Thus, altered numbers of steroid receptors could explain both the lower serum LH plateau after adult castration in the DDT-treated rat and the lack of a difference between serum LH levels in treated and untreated animals which were neonatally castrated and examined as adults (Chapter 4, Section III).

Again, as was the case with the adrenal, additional postulates for mechanisms cannot be excluded. Only further studies will fully illuminate the picture. The initial observations may help guide research on the neonatally dosed male rat by eliminating several approaches from further consideration and by indicating several research directions which may be productive.

1. Measurements of the adult castration response for longer periods of time after castration should determine if a final equilibrium state similar to that of controls does exist.

2. Measurements of LHRH response should be repeated using more than two injections of LHRH to further define the magnitude of the response. Combined injections of LHRH and testosterone or o,p'-DDT might also give clues as to the functional state of the tonic hypothalamic center.

3. Measurements of pituitary release of LH and FSH in vitro in response to LHRH, testosterone and o,p'-DDT should be done to provide data on the comparative sensitivities of the pituitaries from DDT-treated and untreated animals. Such studies could also measure sensitivity to corticosterone and determine basal ACTH secretion.

4. Histochemical or RIA measurements of brain LHRH levels could also be done and would probably reveal much concerning the relative sensitivities of the brains from DDT-treated and untreated animals to stimuli such as exogenous LHRH, testosterone, o,p'-DDT, ether, light, etc.

5. Further studies involving testicular sensitivity to LH or hCG could also be conducted. They must, however, utilize cholesterol as substrate (224). An initial measurement using tritiated acetate of very high specific activity, conducted during these studies, failed to demonstrate any incorporation of label into the isolated

testosterone in either the control tissue, as could have been predicted (224), or the tissue from rats neonatally injected with 10 mg of o,p'-DDT. This result could only imply that the large cholesterol pool (or whatever other mechanism acts to prevent rats from incorporating labelled acetate into testosterone) had not been disturbed sufficiently in DDT-treated rats to allow incorporation of label into testosterone even in the presence of 10 ug/ml hCG.

6. Studies of specific steroidogenic pathways in testes taken from DDT-treated and control groups could be studied. This approach formed the basis for a few *in vitro* experiments during this study which will be discussed in Chapter 6. Briefly stated, they have shown no conclusive effect of neonatal treatment with o,p'-DDT on adult testicular steroid production. In view of the known ability of o,p'-DDT to bind to receptors and the P-450 forms found in the adrenal (223) more data should be collected to test for direct interference of o,p'-DDT (or its metabolites) with steroidogenesis.

If the male rat continues to be an object of study, other types of measurements, including assays for serum androgens, FSH and prolactin, could well be used to monitor hormonal status in much the same manner as has been attempted in this research.

III. Summary:

Further investigation of the parameters studied or proposed in this project may well allow more rapid toxicological screening of materials for effects on the functional status of the steroidogenic endocrine systems. This should also provide support for, if not an alternative means to, predictions of effects on reproductive capacity and viability. Use of the neonatally treated animal is to be recommended when possible because its susceptibility to permanent damage is apparent in these and other studies (53,54,57-61,150,151).

The studies demonstrate the type of data which may be generated in a broad investigation. As a consequence of emphasizing scope and of the time consumed in examining it, depth of study of the positive results has been sacrificed. The projected experiments were designed to measure the systems and tissues which have been demonstrated in this study to be effected by neonatal treatment with o,p'-DDT in more detail.

The whole-animal experiments described demonstrate that neonatal treatment with o,p'-DDT does effect the steroidogenic system of the male rat. It causes change in at least two loci, the immature adrenal cortex and the adult hypothalamo-pituitary axis; such treatment does not, however, grossly affect development. At least part of the effects of the treatment may be due to a metabolite of o,p'-DDT rather than the parent compound. And, the parent compound and at least some of its metabolites are effectively transferred to suckling pups who are, in turn, affected by such naturally administered material.

Though these results seem modest and many other experiments building on them are possible, let it suffice that these experiments serve as a basis for more specifically focused studies which may yield mechanistic information on the mode of o,p'-DDT action, more insight into the control and development of the steroidogenic endocrines and, lastly, more specific rapid and simple testing procedures which may be of use in clinical and environmental monitoring situations.

CHAPTER 6

INVESTIGATION OF Δ^5-PREGNENOLONE CONVERSION IN VITRO

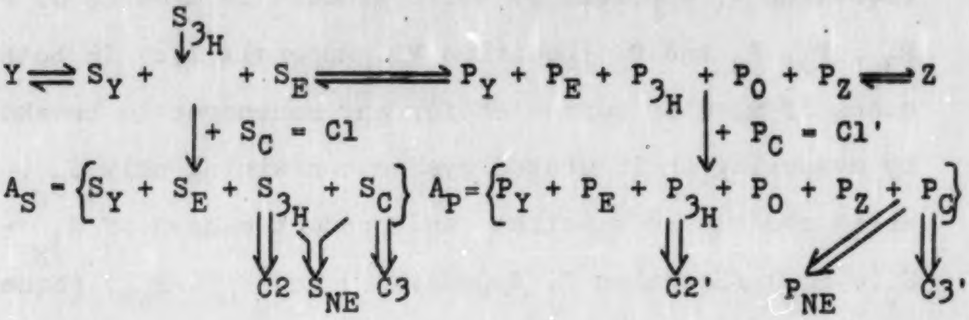
The original conception of this project involved an examination of the mutual interactions of the metabolism of o,p'-DDT and the metabolism of steroids within the testes of animals which had been treated with o,p'-DDT or a vehicle. Since o,p'-DDT had been shown to act as an estrogen (22,24,53,146-148) and to bind to the hepatic P-450 involved in steroid hydroxylations (5,12-15, 18,128,129), and since the closely related metabolite, o,p'-DDD, was known to interfere with steroidogenesis in the adrenal (37,40,42-47), it appeared reasonable to study the interactive metabolisms of DDT and steroids within the testes. That is, it seemed reasonable to attempt to measure the effects of o,p'-DDT and the metabolites which it formed in vitro on the metabolism of Δ^5-pregnenolone to androgens in vitro and to concurrently measure the effects of Δ^5-pregnenolone and the metabolites, e.g., androgens, which it formed in vitro on the metabolism of o,p'-DDT to its metabolites in vitro. To that end incubation procedures, extraction protocols and metabolite assay methods were designed to allow measurements to be done on both sets of metabolites when they were formed in a single incubation mixture. The attractiveness of this combined approach lies in its efficiency in terms of the time, materials and tissues used to generate a set of results which reflect the multiple forms of interaction which might occur in vivo. The procedures used in these studies were developed and improved throughout much of the study; some of them still need further improvements (present versions were described in Chapter 2, Section IV.F.1).

The examination of the metabolisms of two rather different sets of compounds required that some adjustments of the incubation conditions commonly used with each be made to accomodate incubations of both simultaneously. The discrepant time courses for metabolic incubations previously done on the steroids (192,225-227) and DDT analogs (104,113,133) indicated the necessity of measuring steroid and DDT metabolites in aliquots of incubations taken over the course of a reasonable time period (1 hour). Because the potential for DDT interactions with the mitochondrial P-450 was unknown, this subcellular fraction was not discarded before incubations were conducted although its presence for conversions of Δ^5-pregnenolone to androgens was unnecessary (Chapter 1, Section I) (6). The similarity of cofactor requirements for steroid conversions in the testis (6) and for DDT conversions in the liver (113), along with the length of the incubation time, dictated the addition of reduced pyridine nucleotides or systems capable of generating them. Finally, the protein concentrations used appeared to be an adequate compromise between those used previously in steroidal studies (192, 226) and in DDT catabolism studies (104).

The possibility that o,p'-DDT might act at any of several metabolic steps on the pathways from Δ^5-pregnenolone to testosterone brought up the question of the value of measuring intermediates. Since time course data was already to be generated it was decided that several of the known intermediates (progesterone, 17α-hydroxyprogesterone, dehydroepiandrosterone and Δ^4-androstenedione, i.e., products 4 or D, 5 or E, 1 or A and 2 or B in the computer program printout of Appendix I) should be examined at each time point in addition to the substrate and end-product in order to provide a good picture of the metabolic flux from the substrate, Δ^5-pregnenolone (substrate and product 3 or C in the computer printout of Appendix I), to the end-product, testosterone (product 2 or B in the computer printout of Appendix I). Since the metabolism of o,p'-DDT in the testis was unknown the potential production of fairly polar metabolites could not be excluded. The procedures adopted for extraction, however, limited examination of the metabolites of DDT to those which were neutral. Because of this limitation on the number of DDT metabolites which could be examined, because of technical limitations on time available for the entire proposed analysis and because alterations of steroid metabolism due to treatment with DDT were more critical to the focus of the project on altered reproductive capacity, emphasis was placed on examining steroidal flux within the incubation systems given in Table 3 (Chapter 2, Section IV.F.1).

Steroid flux of this type appeared to be best studied by using radioactive substrate (195,228,229). Use of a second label added after stopping active metabolism could provide added information by accounting for losses which occurred during extraction and purification. Similar information, or information additional to it, could also be generated by using a sensitive mass detection system. Since gas chromatography had already been used extensively for measuring steroids (195,228,229), and because it was a method of choice for measuring DDT metabolites, it was chosen as the mass determination system. The GC was also capable of separating a number of steroids simultaneously and, thus, could serve as a separation, as well as a quantitation instrument. Therefore, it seemed reasonable that if radioactivity could be successfully monitored for individual chromatographic peaks it should be possible to generate a series of doubly labelled intermediates and analyze them simultaneously by combined GC-double label scintillation techniques. Since the main object of the overall studies was to analyze the differences between DDT-treated and untreated animals some decreased precision in separations and peak identifications was allowable if samples from incubations of tissues from both types of animals were analyzed similarly.

With this experimental conception in mind a model for the analysis of a single compartment metabolic system emerged.


$S_{{}^3H}$
$Y \rightleftharpoons S_Y +$
$+ S_E \rightleftharpoons P_Y + P_E + P_{{}^3H} + P_O + P_Z \rightleftharpoons Z$
$+ S_C = C1$
$+ P_C = C1'$
$A_S = \{S_Y + S_E + S_{{}^3H} + S_C\}$
$A_P = \{P_Y + P_E + P_{{}^3H} + P_O + P_Z + P_C\}$
C2
S_{NE}
C3
C2'
P_{NE}
C3'

In this model Y is the endogenous precursor for formation of substrate S, and subsequently for the formation of P and Z from S. During the incubation, Y produces amounts of S and P (S_Y and P_Y) which are in addition to the amounts of endogenous S and P (S_E and P_O) which exist prior to the incubation. S_E also forms an amount of product, P_E, during incubation as does Z, any alternate source of product, P_Z. If tritiated substrate $S_{{}^3H}$ is added prior to incubation it also forms product, $P_{{}^3H}$, during the incubation. If a sample of an incubation is stopped at time zero and amounts of carbon-14 substrate and product, S_C and P_C, are added, the total concentration of substrate is due to S_E, $S_{{}^3H}$ and S_C (two of which are known) (Equation III, Appendix I). Similarly, the product contained in the sample would be made up of P_O and P_C (one of which is known) (Equation IV, Appendix I). Because all but one portion of the mass is known apriori a zero time point should allow determination of the mass of endogenous substrates (S_E) (Equation X, Appendix I) and endogenous products, P_O (Equation XI, Appendix I). At times greater than zero substrate is made up of S_Y, S_E, S_{3_H} and S_C (Equation V, Appendix I) while product is made up of P_Y, P_{3_H}, P_O, P_Z and P_C (Equation VI, Appendix I). In both cases if mass is corrected for any nonenzymatic breakdown by measuring an incubated system containing only S_{3_H}, which results in substrate and product masses of S_{3_H} + S_C(= S_{NE}) (Equation I, Appendix I) and P_C (=P_{NE}) (Equation II, Appendix I), the conversion from Y to S_Y and P_Y (Equation XV, Appendix I) and from S_{3_H} and S_E to P_{3_H} and P_E (Equation 2, Appendix I) can be computed. This is done by recalculating the specific activity of the tritiated substrate to include the initial mass of the cold endogenous substrate S_E at time zero or the entire measured mass of the substrate other than S_C at times greater than zero.

Chromatographic data, A_S and A_P, or other measurements of total mass can be combined with the levels of radioactivity (C1, C2, C3, C1', C2', C3') to generate values for mass of the various subsets of total mass of substrate or product. By measuring mass and radioactivity at time zero S_E and P_O are determined as stated above. Radioactivity based on the initial specific activity of S_{3_H} allows computation of the quantities of S_{3_H} and P_{3_H} at any time beyond zero (Equation 2, Appendix I). If the specific activity of S_{3_H} is altered to include S_E then P_E can also be determined (Equation 3, Appendix I). If the specific activity is adjusted to include S_Y as well, P_Y is also determined (Equation 4, Appendix I) and by default, P_Z is generated (Equation XVI, Appendix I). Total net conversions for all forms of substrate or for all forms of product could be measured at any time of incubation by correcting the measured masses for added S_{Cl} (Equation XII, Appendix I) or $P_{Cl'}$ (Equation XIII, Appendix I).

The computer program METFLX, presented in Appendix I, was generated from this model and was subsequently updated to conform to empirical observations. The program presupposes nothing and generates negative concentration values for a product if conversions to later products exceed its formation from endogenous product and all forms of substrate. It also reflects analytical error in the radiochemical assays or mass determinations, especially if too large an amount of cold carrier (added to increase recovery) is used.

The program uses recovery corrections made possible by the added ^{14}C-steroids which were used as internal recovery standards. It bases chromatographic determination of mass on the detector response of known products relative to a chromatographic internal standard and on calibration plots of $\log_{10}$ (relative response) versus $\log_{10}$ (concentration) which were generated from mixtures of all of the compounds of interest and the internal standard. The use of relative peak areas minimizes errors due to misinjection of samples or to slight changes in the sensitivity of the mass detector from injection to injection. It corrects results for individual chromatographic peaks for variations in sample volume injected, in sample size, in efficiency of capillary tube capture, in differences in mass flow to the detector and capture tube, in differences between internal standard concentration in the samples and calibration mixtures, etc. Finally, it bases radioactivity determinations on the specific activity of the added substrate and the computed ^{3}H-dpm and ^{14}C-dpm for captured effluent.

Proof of this program was delayed due to problems involving identification of appropriate GC conditions which would optimize steroid separations and give the cleanest results for the proposed approach. Over thirty single and combined column phases were examined for their ability to separate the steroids which were chosen for study. Parent compounds, silyl-methyl-oxime derivatives, silyl-butyl-oxime derivatives, silyl-benzyl-oxime derivatives, acetate derivatives and benzoate derivatives were examined on one or several of the phases studied. Choice of cholesterol-3-propyl ether as the chromatographic internal standard was made after examining its chromatographic properties with respect to those of both the steroids of interest and 5α-androstane, 5α-pregnane, estradiol, estriol and cholesterol. The standard had the desirable characteristics of being stable, fairly similar in structure to the compounds of interest and of chromatographing in a fashion quite similar to the steroids of interest. A series of modifications of the effluent capture procedure led to the final choice of a simple chilled capillary capture tube. The choice of the GC column conditions and steroid form were a compromise of obtaining workable separations and capture efficiencies without requiring extensive preliminary treatment of the samples to be measured - the parent compounds were separated on a 350 cm x 2 mm column containing Gas Chrom Q coated with 1.5% each of SE-54 and OV-7 by using a temperature gradient of 1°/minute from 175° to 275°C and a flow rate of 25 ml/minute for the carrier gas, nitrogen (Chapter 2, Section IV.F.2). Separations were done using relatively crude extracts containing amounts of carrier steroid, sufficient to yield good recoveries of radioactive steroids throughout isolation and analysis.

By the time these technical matters had been resolved, the whole animal studies were well under way. The generality of the program which had been written made it quite desirable to test it on experimental results even if part of the initial strong impetus to look at direct interactions of DDT and steroids within the testes had somewhat waned. Since the testicular steroid conversions in DDT-treated and untreated animals could still fit into the scope of the overall project and still might generate some insight into either any imprinted changes in steroid metabolism within treated testes or any changes in steroid-DDT interactions within treated tissues, it was decided to test the program and do the originally proposed measurements on the testes from DDT-treated and control animals.

Testes derived from rats exposed neonatally not only to DDT, but also to EV and TP were incubated under the protocol given in Table 3. The incubation mixture aliquots were extracted and stored. Extracts from incubations Type 2 (Δ^5-pregnenolone only) and Type 5 (non-enzymatic incubation) were measured under the conditions listed in Figures 11 and 12 (Chapter 2, Section IV.F.1 and 2). Counting results were normalized to the values which would have been expected for 1 ul injection volumes to allow more rapid pre-program comparisons. Relative peak areas were computed both by means of the fourth revision of an automatic integration program which linked the GC to an electronic calculator and by manual triangulation. Examination of the raw data indicated adequate capture of ^{3}H- and ^{14}C-counts (minimally 50-100 dpm/ul above chromatographic background counts for each radioactive label) and good agreement between automatic and manual integration for relative areas for all but the 17α-hydroxyprogesterone peak, which only manual integration measured. Plots of the ^{3}H/^{14}C ratio (not shown) for each of the steroid peaks in the chromatograms from the testicular incubations of control animals (neonatally injected with sesame oil) indicated the predicted pattern of ^{3}H-labelling.

Tritiated substrate declined to essentially zero levels by 30 minutes of incubation. The products of both the Δ^4- and Δ^5-pathways, testosterone and Δ^4-androstenedione, rose beyond 5-10 minutes of incubation until essentially all the ^{3}H-label was incorporated into them by 30-60 minutes. Progesterone and 17α-hydroxyprogesterone rose to a peak by 10 minutes of incubation and declined to baseline by 30 minutes. Dehydroepiandrosterone (or the labelled compound associated with that chromatographic peak - Chapter 2, Section IV.F.2) rose slowly after 10-30 minutes of incubation to a level of about 1/5 that seen for testosterone plus Δ^4-androstenedione. (This agreement of the radioactivity pattern for dehydroepiandrosterone with the predicted pattern tends to support the identity of the label in this chromatographic effluent with the carrier steroid.) Analogous plots for testicular incubations done on tissues from rats neonatally injected with EV (200 ug total), TP (1000 ug total) or o,p'-DDT (500 ug total) indicated somewhat similar profiles.

METFLX was run using the results from the incubation of all the treatment groups. The resultant data are presented in Appendix I, as an example of the program printout.

The plots shown at the end of the printout illustrate conversion of the ^{3}H-label and demonstrate patterns similar to the plots obtained from ^{3}H-/^{14}C- ratios, as expected. It can be seen in the second plot (Treatment II) that steroid metabolism in EV-treated animals appears more rapid than in the controls; this may, however, be due to a larger number of Leydig cells per gram of testicular weight than in the control (62) as may also be the case in the third plot (Treatment III, TP treatment). Both sets of results, however, agree with the findings of Joseph and Kincl (230) in similarly treated rats that metabolic capability is preserved in the testis of such animals even though spermatogenesis is largely halted. The fourth plot (Treatment IV) which describes the situation for DDT-treated animals appears different in that the metabolic conversions appear much slower. This may, however, be an artifact due to inadequate aeration of the incubation since a larger incubation volume was used for this tissue; it was incubated somewhat earlier than the steroid and control tissues. Still, it may also indicate a reduced rate of steroid synthesis in treated animals and with the results from the whole animal studies imply an overall sluggishness of response within the hypothalamo-pituitary-testicular axis of DDT-treated rats.

Other data given in the program printout show masses which reflect the amount of carrier steroids (about 300 nmoles each) which had been added during the isolation procedure. It is obvious that this mass is too large to allow good estimation of many of the parameters the program was designed to calculate. An attempt to measure the steroids in this system by using a very large incubation but no carrier steroid demonstrated that GC-measurement using the FID detection system was possible, but that the extract would have to be more rigorously purified prior to injection onto the column in order to allow the accurate manual capture of the desired effluents.

The approach at this stage appears usable if a few modifications are made; the program seems to function very well, generating accurate results for the desired parameters and being limited only by the precision of the values on which it must act. Modifications of this approach which could make it quite useful in the area of steroid metabolism and/or comparative steroid metabolism (as here) would be: 1) to move to the use of electron capture detection of appropriate derivatives to allow precise measurements of very small amounts of steroids; 2) to use minimal amounts of carrier, e.g. 1-10 ng per steroid, to decrease artifactual change due to deviations in carrier concentration, 3) to interpose TLC, lipophilic Sephadex chromatography or some other purification and/or steroid-group separation between extraction and GC analysis to assist in decreasing both chemical and radioactivity background, and 4) to use capillaries filled with terphenyl crystals to optimize capture efficiencies so that smaller amounts of ^{14}C-carrier and/or ^{3}H- could be accurately detected in the GC effluents (195,228,229). Potential major modifications of the methodology used in the present study have evolved elsewhere during the course of these studies so that now it may be feasible to use GC-mass spectral equipment in combination with Fourier transform techniques to measure mass and isotope ratios simultaneously. Or, RIA might be used for determination of the masses of most of the steroids involved.

The attractiveness of using an approach such as the one used in these studies is that when fully developed it can yield data not only on conversion of labelled substrate but also on conversion of other forms of substrate and on sizes of metabolic pools. Presently, such data are generated by doing a series of several experiments, many times a separate set for each individual product or intermediate. Obviously, for such a limited approach to be useful in clinical or environmental monitoring the tests must be quite rapid. The approach which was proposed and tested in these studies would be perhaps more rapid by generating all the data for all the compounds of interest in a somewhat longer single procedure.

The attractiveness of the program lies in its generality. It may be used to generate solely mass or radioactivity data or a combination of both; it may be used to calculate data from any combination of four incubations - 2 test groups and 2 controls, 4 different buffers, etc.; and it may be used to compute metabolic flux through a single compartment system for any of a large variety of compounds, e.g., amino acids, sugars, drugs, etc. In drug studies as an example, it can even incorporate measurements of chemically unidentified intermediates as long as some sort of standard curve can be generated from which masses (or absorbance units, etc.) can be computed.

Measurements on the steroids and DDT metabolites from incubation Types 1, 3 and 4 (Table 3) can still be run on the samples which were collected during this investigation though their results must be viewed as preliminary until they can be repeated using one or more of the suggested modifications in the isolation, purification and measurement protocols. Measurements of other types of incubation, e.g., incorporating hCG and ^{3}H-cholesterol in DDT-treated and control testicular incubations could well extend the results and spirit of the present investigation.

Contributions of these methodological studies were twofold: 1) a method of measuring doubly-labelled steroids in GC effluents, which was proposed earlier as a desirable objective by Eik-Nes (195) for samples containing single compounds, and 2) a FORTRAN program, METFLX, which can analyze results of the type generated in this study but can also be used in other studies of rather varied forms.

LIST OF REFERENCES

Note: For brevity when the full chemical names of DDT analogs appear in article titles they have been replaced by appropriate abbreviations, e.g., 2-(2-chlorophenyl)-2-(4-chlorophenyl)-1,1,1-trichloroethane appears as [o,p'-DDT].

1. Tomkins, G. M., Gelehrter, T. D., Granner, D., Martin, D., Jr., Sammuels, H. H. and Thompson, E. B. Control of specific gene expression in higher organisms. Science 166 (1969) 1474-1480.
2. O'Malley, B. W., Sherman, M. R. and Toff, D. O. Progesterone "Receptors" in the cytoplasm and nucleus of chick oviduct target tissue. Proc. Nat. Acad. Sci. 67 (1970) 501-508.
3. Schally, A. V., Kastin, A. J. and Arimura, A. The hypothalamus and reproduction. Am. J. Obstet. Gynecol. 114 (1972) 423-442.
4. Schriefers, H. Factors regulating the metabolism of steroids in the liver. Adv. in the Biosciences 2 (1969) 69-90.
5. Conney, A. H. Pharmacological implications of microsomal enzyme induction. Pharm. Rev. 19 (1967) 317-366.
6. Tamaoki, B.-I. Steroidogenesis and cell structure. J. Steroid Biochem. 4 (1973) 89-118.
7. Rasmussen, H. Ions as 'Second Messangers', Chapter 20. In Cell Membranes, 203-212. Ed. G. Weissmann and R. Claiborne. New York: H. P. Publishing Co., Inc., 1975.
8. Cooke, B. A., vander Molen, H. J. and Setchell, B. P. The testis. Research in Reproduction 5 (1973).
9. Schally, A. V., Arimura, A. and Kastin, A. J. Hypothalamic regulatory hormones. Science 179 (1973) 341-350.
10. Frieden, E. and Lipner, H. Biochemical endocrinology of the vertebrates. Englewood Cliffs, New Jersey: Prentice-Hall, Inc., 1971.
11. Conney, A. H., Schneidman, K., Jacobson, M. and Kuntzman, R. Drug-induced changes in steroid metabolism. Ana. N.Y. Acad. Sci. 123 (1965) 98-109.

12. Kuntzman, R., Sansur, M. and Conney, A. H. Effect of drugs and insecticides on the anesthetic action of steroids. *Endocrinology* *77* (1965) 952-954.

13. Conney, A. H., Jacobson, M., Levin, W., Schneidman, K. and Kuntzman, R. Decreased central depressant effect of progesterone and other steroids in rats pretreated with drugs and insecticides. *J. Pharmacol. Exp. Therap.* *154* (1966) 310-318.

14. Welch, R. M., Levin, W. and Conney, A. H. Insecticide inhibition and stimulation of steroid hydroxylases in rat liver. *J. Pharmacol. Exp. Therap.* *155* (1967) 167-173.

15. Conney, A. H., Welch, R. M., Kuntzman, R. and Burns, J. J. Effects of pesticides on drug and steroid metabolism. *Clin. Pharmacol. Therap.* *8* (1967) 2-10.

16. Welch, R. M., Levin, W. and Conney, A. H. Stimulatory effect of phenobarbital on the metabolism *in vivo* of estradiol-17β and estrone in the rat. *J. Pharmacol. Therap.* *160* (1968) 171-178.

17. Levin, W., Welch, R. M. and Conney, A. H. Effect of carbon tetrachloride and other inhibitors of drug metabolism on the metabolism and action of estradiol-17β and estrone in the rat. *J. Pharmacol. Therap.* *173* (1970) 247-255.

18. Welch, R. M., Levin, W., Kuntzman, R., Jacobson, M. and Conney, A. H. Effect of halogenated hydrocarbon insecticides on the metabolism and uterotropic action of estrogens in rats and mice. *Toxicol. Appl. Pharmacol.* *19* (1971) 234-246.

19. Levin, W., Welch, R. M. and Conney, A. H. Effect of chronic phenobarbital treatment on the liver microsomal metabolism and uterotropic action of 17β -estradiol. *Endocrinology* *80* (1967) 135-140.

20. Levin, W., Welch, R. M. and Conney, A. H. Effect of phenobarbital and other drugs on the metabolism and uterotropic action of estradiol-17β and estrone. *J. Pharmacol. Exp. Therap.* *159* (1968) 362-371.

21. Levin, W., Welch, R. M. and Conney, A. H. Decreased uterotropic potency of oral contraceptives in rats pretreated with phenobarbital. *Endocrinology* *83* (1968) 149-156.

22. Welch, R. M., Levin, W. and Conney, A. H. Estrogenic action of DDT and its analogs. *Toxicol. Appl. Pharmacol*. 14 (1969) 358-367.

23. Levin, W., Welch, R. M. and Conney, A. H. Inhibitory effect of phenobarbital or chlordane pretreatment on the androgen-induced increase in seminal vesicle weight in the rat. *Steroids* 13 (1969) 155-161.

24. Bitman, J. and Cecil, H. C. Estrogenic activity of DDT analogs and polychlorinated biphenyls. *J. Agr. Food Chem*. 18 (1970) 1108-1112.

25. Smith, M. T., Thomas, J. A., Smith, C. G., Mawhinney, M. G. and Lloyd, J. W. Effects of DDT on radioactive uptake from testosterone-1,2-^{3}H by mouse prostate glands. *Toxicol. Appl. Pharmacol*. 23 (1972) 159-164.

26. Nelson, J. A. Effects of dichlorodiphenyltrichloroethane (DDT) analogs and polychlorinated biphenyl (PCB) mixtures on 17β - ^{3}H - estradiol binding to rat uterine receptor. *Biochem. Pharmac*. 23 (1974) 447-451

27. Foster, M. S., Gellert, R. J. and Heinrichs, W. L. The estrogenic capacity of organochlorine pesticides. *Soc. Gyn. Invest*. (Abs.) 5 (1974) 35-36.

28. Rybakova, M. N. Comparative toxic effects of Sevin and DDT used in the treatment of food crops. *Voprosy Pitaniia* 26 (1967) 9-15. Translated by A. Nichols.

29. Rybakova, M. N. About several pesticide effects on the hypophysis and its gonadotrophic function. *Gigiena I Sanitariia* 33 (1969) 27-31. Translated by L. Ratermanis and M. Mizens.

30. Shtenberg, A. I. and Rybakova, M. N. Effect of carbaryl on the neuroendocrine system of rats. *Fd. Cosmet. Toxicol*. 6 (1968) 461-467.

31. Ottoboni, A. Effect of DDT on the reproductive life-span in the female rat. *Toxicol. Appl. Pharmacol*. 22 (1972) 497-502.

32. Ware, G. W. and Good, E. E. Effects of insecticides on reproduction in the laboratory mouse. II. Mirex, telodrin and DDT. *Toxicol. Appl. Pharmacol*. 10 (1967) 54-61.

33. Harr, J. R., Claeys, R. R., Bone, J. F. and McCorcle, T. W. Dieldrin toxicosis: rat reproduction. *Am. J. Vet. Res*. 31 (1970) 181-189.

34. Beattie, C. W., Campbell, C. S., Nequin, L. G., Soyka, L. F. and Schwartz, N. B. Barbiturate blockade of tonic LH secretion in the male and female rat. Endocrinology 92 (1973) 1634-1638.

35. Ajika, K., Kalra, S. P., Fawcett, C. P., Krulich, L. and McCann, S. M. The effect of stress and nembutal on plasma levels of gonadotropins and prolactin in ovariectomized rats. Endocrinology 90 (1972) 707-715.

36. Howland, B. E., Beaton, D. B. and Jack, M. I. Changes in serum levels of gonadotropins and testosterone in the male rat in response to fasting, surgery and ether. Experimentia 30 (1974) 1223-1225.

37. Gallagher, T. F., Fukushima, D. K. and Hellman, L. The effect of ortho,para'DDD on steroid hormone metabolites in adrenocortical carcinoma. Metabolism 11 (1962) 1155-1161.

38. Bradlow, H. L., Fukushima, D. K., Zumoff, B., Hellman, L. and Gallagher, T. F. A peripheral action of o,p'-DDD on steroid biotransformation. J. Clin. Endocr. 23 (1963) 918-922.

39. Bledsoe, T., Island, D. P., Ney, R. L. and Liddle, G. W. An effect of o,p'-DDD on the extra-adrenal metabolism of cortisol in man. J. Clin. Endocr. 24 (1964) 1303-1311.

40. Southern, A. L., Tochimoto, S., Strom, L., Ratuschni, A., Ross, H. and Gordon, G. Remission in Cushing's syndrome with o,p'-DDD. J. Clin. Endocr. 26 (1966) 268-278.

41. Southern, A. L., Tochimoto, S., Isurugi, K., Gordon, G., Krikun, E. and Stypulkowski, W. The effect of [o,p'-DDD] on the metabolism of infused cortisol-7-^{3}H. Steroids 7 (1966) 11-29.

42. Nelson, A. A. and Woodard, G. Severe adrenal atrophy (cytotoxic) and hepatic damage produced in dogs by feeding [p,p'-DDD]. Arch. Pathol. 48 (1949) 387-394.

43. Cobey, F., Taliaferro, I. and Haag, H. B. Effect of DDD and some of its derivatives on plasma 17-OH-corticosteroids in the dog. Science 123 (1956) 140-141.

44. Nichols, J. and Hennigar, G. Studies on [DDD]. Exp. Med. Surg. 15 (1957) 310-316.

45. Cueto, C., Brown, J. H. U. and Richardson, A. P. Biological studies on an adrenocorticolytic agent and the isolation of the active components. Endocrinology 62 (1958) 334-339.

46. Vilar, O. and Tullner, W. W. Effects of o,p'-DDD on histology and 17-hydroxycortico-steroid output of the dog adrenal cortex. Endocrinology 65 (1959) 80-86.

47. Kaminsky, N., Luse, S. and Hartroft, P. Ultrastructure of adrenal cortex of the dog during treatment with DDD. J. Nat. Cancer Inst. 29 (1962) 127-159.

48. Kupfer, D., Balazs, T. and Buyske, D. A. Stimulation by o,p'-DDD of cortisol metabolism in the guinea pig. Life Sciences 3 (1964) 959-964.

49. Balazs, T. and Kupfer, D. Effect of DDT on the metabolism and production rate of cortisol in the guinea pig. Toxicol. Appl. Pharmacol. 9 (1966) 40-43.

50. Foster, T. S. Effect of some pesticides on the adrenal glands in the rat. Can. J. Biochem. 46 (1968) 1115-1120.

51. Reif, V. D. and Sinsheimer, J. E. Metabolism of [o,p'-DDD] in rats. Drug Metab. Disp. 3 (1975) 15-25.

52. Hiddemen, J. W. III. "The effects of dieldrin and tolbutamide on exogenous and endogenous glucose in the rat." Ph.D. Dissertation, The University of Michigan, 1970.

53. Heinrichs, W. L., Gellert, R. J., Bakke, J. L. and Lawrence, N. L. DDT administered to neonatal rats induces persistent estrus syndrome. Science 173 (1971) 642-643.

54. Gellert, R. J., Heinrichs, W. L. and Swerdloff, R. Effects of neonatally-administered DDT homologs on reproductive function in male and female rats. Neuroendocrinology 16 (1974) 84-94.

55. Moffenson, H. C., Greensher, J. and Horowitz, R. Hazards of maternally administered drugs. Clin. Toxicol. 7 (1974) 59-68.

56. Knowles, J. A. Breast milk: a source of more than nutrition for the neonate. Clin. Toxicol. 7 (1974) 69-82.

57. Harris, G. W. Sex hormones, brain development and brain function. Endocrinology 75 (1964) 627-648.

58. Barraclough, C. A. Modifications in the CNS regulation of reproduction after exposure of prepubertal rats to steroid hormones. *Rec. Prog. Hormone Res.* 22 (1966) 503-539.

59. Barraclough, C. A. Modifications in reproductive function after exposure to hormones during the prenatal and early postnatal period, Chapter 19. In *Neuroendocrinology*, Vol. II, 62-99. Ed. L. Martini and W. F. Ganong. New York and London: Academic Press, 1967.

60. Barraclough, C. A. Alterations in reproductive function following prenatal and early postnatal exposure to hormones. *Adv. Reprod. Physiol.* 3 (1968) 81-112.

61. Gorski, R. A. Gonadal hormones and the perinatal development of neuroendocrine function, Chapter 9. In *Frontiers in Neuroendocrinology*, 1971, 237-290. Ed. W. F. Ganong and L. Martini. New York: Oxford Univ. Press, 1971.

62. Kincl, F. A., Folch Pi, A. and Lasso, L. H. Effect of estradiol benzoate treatment in the newborn male rat. *Endocrinology* 7 (1963) 966-968.

63. Dörner, G., Döcke, F. and Hinz, G. Paradoxical effects of estrogen on brain differentiation. *Neoroendocrinology* 7 (1971) 146-166.

64. Einarsson, K., Gustafsson, J.-Å. and Stenberg, Å. Neonatal imprinting of liver microsomal hydroxylation and reduction of steroids. *J. Biol. Chem.* 248 (1973) 4987-4997.

65. Berg, A. and Gustafsson, J.-Å. Regulation of hydroxylation of 5α-androstane-$3\alpha,17\beta$-diol in liver microsomes from male and female rats. *J. Biol. Chem.* 248 (1973) 6559-6567.

66. Gustafsson, J.-Å. and Ingelman-Sundberg, M. Regulation and properties of a sex-specific hydroxylase system in female rat liver microsomes active on steroid sulfates. I. General characteristics. *J. Biol. Chem.* 249 (1974) 1940-1945.

67. Gustafsson, J.-Å. and Ingelman-Sundberg, M. Age-dependent induction and repression of rat liver microsomal hydroxylase systems by estradiol. *Biochim. Biophys. Acta* 354 (1974) 172-181.

68. Gustafsson, J.-Å. and Stenberg, Å. Specificity of neonatal, androgen-induced imprinting of hepatic steroid metabolism in rats. Science 191 (1976) 203-204.

69. Flerkó, B., Mess, B. and Illei-Donhoffer, A. On the mechanism of androgen sterilization. Neuroendocrinology 4 (1969) 164-169.

70. McGuire, J. L. and Lisk, R. D. Oestrogen receptor in androgen or oestrogen sterilized female rats. Nature 221 (1969) 1068-1069.

71. Simmons, J. E. Uptake of [1,2-^{3}H]-testosterone in oestrogenized male rats. Acta Endocrinologica 67 (1971) 535-543.

72. Bullock, L. P. and Bardin, C. W. In vivo and in vitro testosterone metabolism by the androgen insensitive rat. J. Steroid. Biochem. 4 (1973) 139-151.

73. Mennin, S. P., Kubo, K. and Gorski, R. A. Pituitary responsiveness to luteinizing hormone-releasing factor in normal and androgenized female rats. Endocrinology 95 (1974) 412-416.

74. Niemi, M. and Ikonen, M. Histochemistry of the Leydig cells in the postnatal prepubertal testis of the rat. Endocrinology 72 (1963) 443-448.

75. Clermont, Y. and Perey, B. Quantitative study of the cell population of the seminiferous tubules in immature rats. Am. J. Anat. 100 (1957) 241-260.

76. Warren, D. W., Haltmeyer, G. C. and Eik-Nes, K. B. The effect of gonadotrophins on the fetal and neonatal rat testis. Endocrinology 96 (1975) 1226-1229.

77. Döhler, K. D. and Wuttke, W. Changes with age in levels of serum gonadotropins, prolactin, and gonadal steroids in prepubertal male and female rats. Endocrinology 97 (1975) 898-907.

78. Engel, W. and Frowein, J. Glucocorticoids and hCG sensitivity of rat testicular Leydig cell. Nature 251 (1974) 146-148.

79. Frowein, J. and Engel, W. Constitutivity of the HCG-receptor protein in the testis of rat and man. Nature 249 (1974) 377-379.

80. Resko, J. A., Feder, H. H. and Goy, R. W. Androgen concentrations in plasma and testis of developing rats. *J. Endocr*. *40* (1968) 485-491.

81. Knorr, D. W., Vanha-Perttula, T. and Lipsett, M. B. Structure and function of rat testis through pubescence. *Endocrinology 86* (1970) 1298-1304.

82. Miyachi, Y., Nieschlag, E. and Lipsett, M. B. The secretion of gonadotropins and testosterone by the neonatal male rat. *Endocrinology 92* (1973) 1-5.

83. Payne, A. H., unpublished results.

84. Araki, S., Toran-Allerand, C. D., Ferin, M. and Vande Wiele, R. L. Immunoreactive gonadotropin-releasing hormone (Gn-RH) during maturation in the rat: ontogeny of regional hypothalamic differences. *Endocrinology 97* (1975) 693-697.

85. Goldman, B. D., Grazia, Y. R., Kamberi, I. A. and Porter, J. C. Serum gonadotropin concentrations in intact and castrated neonatal rats. *Endocrinology 88* (1971) 771-776.

86. Eldridge, J. C., Dmowski, W. P. and Mahesh, V. B. Effects of castration of immature rats on serum FSH and LH, and of various steroid treatments after castration. *Biol. Reprod*. *10* (1974) 438-446.

87. Korenbrot, C. C., Paup, D. C. and Gorski, R. A. Effects of testosterone propionate and dihydrotestosterone propionate on plasma FSH and LH levels in neonatal rats and on sexual differentiation of the brain. *Endocrinology 97* (1975) 709-717.

88. Peters, H. The development of the mouse ovary from birth to maturity. *Acta Endocrinologia 62* (1969) 98-116.

89. Midgley, A. R., Jr., Zeleznik, A. J., Richards, J. S. and Han, S. S. Gonadotropin and estradiol receptors and follicular development. In *Advances in Fertility Regulation Through Basic Research*. Ed. W. A. Sadler and S. Segal. New York: Plenum Press, 1974. Preprint.

90. Baker, F. D. and Kragt, C. L. Maturation of the hypothalamic-pituitary-gonadal negative feedback system. *Endocrinology 85* (1969) 522-527.

91. Ojeda, S. R., Wheaton, J. E., Jameson, H. E. and McCann, S. M. The onset of puberty in the female rat: changes in plasma prolactin, gonadotropins, luteinizing hormone-releasing hormone (LHRH), and hypothalamic LHRH content. Endocrinology 98 (1976) 630-638.

92. Swerdloff, R. S., Walsh, P. C., Jacobs, H. S. and Odell, W. D. Serum LH and FSH sexual maturation in the male rat: effect of castration and cryptorchidism. Endorcinology 99 (1971) 120-128.

93. Odell, W. D., Swerdloff, R. S., Jacobs, H. S. and Hescox, M. A. FSH induction of sensitivity to LH: one cause of sexual maturation in the male rat. Endocrinology 92 (1973) 160-165.

94. Howard, E. The representation of the adrenal x zone in rats, in the light of observations on x zone variability in mice. Am. J. Anat. 62 (1938) 351-373.

95. Milković, K. and Milković, S. Functioning of the pituitary-adrenocortical axis in rats at and after birth. Endocrinology 73 (1963) 535-539.

96. Haltmeyer, G. C., Denenberg, V. H., Thatcher, J. and Zarrow, M. X. Response of the adrenal cortex of the neonatal rat after subjection to stress. Nature 212 (1966) 1371-1373.

97. Eguchi, Y., Hirai, O., Morikawa, Y. and Hashimoto, Y. Critical time in hypothalamic control of the pituitary-adrenal system in fetal rats: observations in fetuses subjected to hypervitaminosis A and hypothalamic destruction. Endocrinology 93 (1973) 1-11.

98. Gorski, M. E. and Lawton, I. E. Adrenal involvement in determining the time of onset of puberty in the rat. Endocrinology 93 (1973) 1232-1234.

99. Haller, H. L., Bartlett, P. D., Drake, N. L., Newman, M. S., Cristol, S. J., Eaker, C. M., Hayes, R. A., Kilmer, G. W., Magerlein, B., Mueller, G. P., Schneider, H. and Wheatley, W. The chemical composition of technical DDT. J. Am. Chem. Soc. 67 (1945) 1591-1602.

100. Peterson, J. E. and Robison, W. H. Metabolic products of p,p'-DDT in the rat. Toxicol. Appl. Pharmacol. 6 (1964) 321-327.

101. Pinto, J. D., Camien, M. N. and Dunn, M. S. Metabolic fate of [p,p'-DDT] in rats. J. Biol. Chem. 240 (1965) 2148-2154.

102. Miskus, R. P., Blair, D. P. and Casida, J. E. Conversion of DDT and DDD by bovine rumen fluid, lake water, and reduced porphyrins. J. Agr. Food Chem. 13 (1965) 481-483.

103. Glass, B. L. Relation between the degradation of DDT and the iron redox system in soils. J. Agr. Food Chem. 20 (1972) 324-327.

104. Hassall, K. A. and Forrest, T. J. Reductive dechlorination of DDT by heated liver. Nature New Biology 236 (1972) 214-216.

105. Hayes, W. J., Jr. Review of the metabolism of chlorinated hydrocarbon insecticides especially in mammals. Ann. Rev. Pharmacol. 5 (1965) 27-52.

106. Durham, W. F. The influence of other factors on the toxicity of pesticides, Chapter 23. In Chemical Fallout, 433-446. Ed. M. W. Miller and G. G. Berg. Springfield, Ill.: Charles C. Thomas, 1969.

107. Durham, W. F. The interaction of pesticides with other factors. Residue Reviews 18 (1967) 21-103.

108. Dale, W. E., Gaines, T. B. and Hayes, W. J., Jr. Storage and excretion of DDT in starved rats. Toxicol. Appl. Pharmacol. 4 (1962) 89-106.

109. Gillett, J. W. Microsomal epoxidation: effect of age and duration of exposure to dietary DDT on induction. Bull. Environ. Contam. Toxicol. 4 (1969) 159-168.

110. Boyd, E. M. and Krijnen, C. J. Dietary protein and DDT toxicity. Bull. Environ. Contam. Toxicol. 4 (1969) 256-261.

111. Wagstaff, D. J. and Street, J. C. Ascorbic acid deficiency and induction of hepatic microsomal hydroxylative enzymes by organochlorine pesticides. Toxicol. Appl. Pharmacol. 19 (1971) 10-19.

112. Chadwick, R. W., Linko, R. S., Freal, J. J. and Robbins, A. L. The effect of age and long-term low-level DDT exposure on the response to enzyme induction in the rat. Toxicol. Appl. Pharmacol. 31 (1975) 469-480.

113. Morello, A. Induction of DDT-metabolizing enzymes in microsomes of rat liver after administration of DDT. Can. J. Biochem. 43 (1965) 1289-1293.

114. Sánchez, E. DDT-induced metabolic changes in rat liver. Can. J. Biochem. 45 (1967) 1809-1817.

115. Hart, L. G. and Fouts, J. R. Effects of acute and chronic DDT administration on hepatic microsomal drug metabolism in the rat. Proc. Soc. Exp. Biol. Med. 114 (1963) 388-392.

116. Hart, L. G. and Fouts, J. R. Further studies on the stimulation of hepatic microsomal drug metabolizing enzymes by DDT and its analogs. Naunyn-Schmiedebergs Arch. Exp. Path. Pharmak. 249 (1965) 486-500.

117. Straw, J. A., Waters, I. W. and Fregly, M. J. Effect of o,p'-DDD on hepatic metabolism of phenobarbital in rats. Proc. Soc. Exp. Biol. Med. 118 (1965) 391-394.

118. Kupfer, D. and Peets, L. The effect of o,p'-DDD on cortisol and hexobarbital metabolism. Biochem. Pharmacol. 15 (1966) 573-581.

119. Azarnoff, D. L., Grady, H. J. and Svoboda, D. J. The effect of DDD on barbiturate and steroid-induced hypnosis in the dog and rat. Biochem. Pharmacol. 15 (1966) 1985-1993.

120. Kuntzmann, R., Jacobson, M., Schneidman, K. and Conney, A. H. Similarities between oxidative drug-metabolizing enzymes and steroid hydroxylases in liver microsomes. J. Pharmacol. Exp. Therap. 146 (1964) 280-285.

121. Platt, O. S. and Cockrill, B. L. Biochemical changes in rat liver in response to treatment with drugs and other agents - II. Effects of Halothane, DDT, other chlorinated hydrocarbons, thioacetamide, dimethylnitrosamine and ethionine. Biochem. Pharmacol. 18 (1969) 445-457.

122. Nowicki, H. G. and Norman, A. W. Enhanced hepatic metabolism of testosterone, 4-androstene-3,17-dione, and estradiol-17β in chickens pretreated with DDT and PCB. Steroids 19 (1972) 85-99.

123. Alary, J. G., Brodeur, J., Côté, M. G., Panisset, J. C., Lamothe, P. and Guay, P. The effect of a pretreatment with phenobarbital on the metabolism of DDT in dairy cows and young bulls. Rev. Can. Biol. 27 (1968) 269-271.

124. Street, J. C. DDT antagonism to dieldrin storage in adipose tissue of rats. Science 146 (1964) 1580-1581.

125. Nowicki, H. G., Wong, R. G., Myrtle, J. F. and Norman, A. W. Inhibition of biological activity of cholecalciferol...by o,p'-DDT or p,p'-DDT in rachitic cockerel. J. Agr. Food Chem. 20 (1972) 376-380.

126. Novicki, H. G., Myrtle, J. F. and Norman, A. W. Effects of organochlorine insecticides on metabolism of cholecalciferol (vitamin D$_3$) in rachitic cockerel. J. Agr. Food Chem. 20 (1972) 380-384.

127. Conney, A. H. and Kuntzman, R. Metabolism of normal body constituents by drug-metabolizing enzymes in liver microsomes. In Handbook of Experimental Pharmacology 28/2, 401-421. Ed. B. B. Brodie and J. Gillette. New York: Springer-Verlag, 1971.

128. Conney, A. H., Welch, R., Kuntzman, R., Chang, R., Jacobson, M., Munro-Faure, A. D., Peck, A. W., Bye, A., Poland, A., Poppers, P. J., Finster, M. and Wolff, J. A. Effects of environmental chemicals on the metabolism of drugs, carinogens, and normal body constituents in man. Ann. N.Y. Acad. Sci. 179 (1971) 155-172.

129. Conney, A. H. and Burns, J. J. Metabolic interactions among environmental chemicals and drugs. Science 178 (1972) 576-586.

130. McCully, K. A., McKinley, W. P. and Phillips, W. E. J. The effect of the route of administration of single doses of p,p'-DDT on residues in adipose and liver tissues. J.A.O.A.C. 51 (1968) 1050-1057.

131. McCully, K. A., Villeneuve, D. C., McKinley, W. P., Phillips, W. E. J. and Hidiroglou, M. Metabolism and storage of DDT in beef cattle. J.A.O.A.C. 49 (1966) 966-973.

132. Plimmer, J. R., Kearney, P. C. and Von Endt, D. W. Mechanism of conversion of DDT to DDD by Aerobacter aerogenes. J. Agr. Food Chem. 16 (1968) 594-597.

133. Sink, J. D., Varela-Alvarez, H. and Hess, C. Metabolism of ^{14}C-DDT by ovine rumen fluid in vitro. J. Agr. Food Chem. 20 (1972) 7-9.

134. Laug, E. P., Nelson, A. A., Fitzhugh, O. G. and Kunze, F. M. Liver cell alteration and DDT storage in the fat of the rat induced by dietary levels of 1 to 50 p.p.m. DDT. J. Pharmacol. Exp. Therap. 98 (1950) 268-273.

135. Bailey, S., Bunyan, P. J., Rennison, B. D. and Taylor, A. The metabolism of [p,p'-DDT] and [p,p'-DDD] in the pigeon. Toxicol. Appl. Pharmacol. 14 (1969) 13-22.

136. Bailey, S., Bunyan, P. J., Rennison, B. D. and Taylor, A. The metabolism of [p,p'-DDE] and [p,p'-DDMU] in the pigeon. Toxicol. Appl. Pharmacol. 14 (1969) 23-32.

137. Feil, V. J., Lamoureux, C. H., Styrovoky, E., Zaylskie, R. G., Thacker, E. J., and Holman, G. M. Metabolism of o,p'-DDT in rats. J. Agr. Food Chem. 21 (1973) 1072-1078.

138. Reif, V. D., Sinsheimer, J. E., Ward, J. C. and Schteingart, D. E. Aromatic hydroxylation and alkyl oxidation in metabolism of Mitotane (o,p'-DDD) in humans. J. Pharmaceutical Sciences 63 (1974) 1730-1736.

139. Cranmer, M. F. Absence of conversion of o,p'-DDT to p,p'-DDT in the rat. Bull. Environ. Contam. Toxicol. 8 (1972) 121-124.

140. Gaines, T. B. Acute toxicity of pesticides. Toxicol. Appl. Pharmacol. 14 (1969) 515-534.

141. Metcalf, R. L. Organic Insecticides. New York: Interscience Pub. Inc., 1955.

142. Hrdina, P. D., Singhal, R. L., Peters, D. A. V. and Ling, G. M. Role of brain acetylcholine and dopamine in acute neurotoxic effects of DDT. Eur. J. Pharmacol. 15 (1971) 379-382.

143. Hrdina, P. D., Singhal, R. L. and Ling, G. M. DDT and related chlorinated hydrocarbon insecticides: pharmacological basis of their toxicity in mammals. Adv. Pharmacol. Chemotherapy 12 (1975) 31-88.

144. Burlington, H. and Lindeman, V. F. Effect of DDT on testes and secondary sex characters of white Leghorn cockerels. Proc. Soc. Exp. Biol. Med. 74 (1950) 48-57.

145. Fischer, A. L., Keasling, H. H. and Schueler, F. W. Estrogenic action of some DDT analogues. Proc. Soc. Exp. Biol. Med. 81 (1952) 439-441.

146. Bitman, J., Cecil, H. C., Harris, S. J. and Fries, G. F. Estrogenic activity of o,p'-DDT in the mammalian uterus and avian oviduct. Science 162 (1968) 371-372.

147. Cecil, H., Bitman, J. and Harris, S. J. Estrogenicity of o,p'-DDT in rats. J. Agr. Food Chem. 19 (1971) 61-65.

148. Singhal, R. L., Valadares, J. R. E. and Schwark, W. S. Metabolic control mechanism in mammalian systems-IX. Estrogen-like stimulation of uterine enzymes by [o,p'-DDT]. Biochem. Pharmacol. 19 (1970) 2145-2155.

149. Quinby, G. E., Hayes, W. J., Armstrong, J. F. and Durham, W. F. DDT storage in the U.S. population. J. Am. Med. Assoc. 191 (1965) 109-113.

150. Gellert, R. J., Heinrichs, W. L. and Swerdloff, R. S. DDT homologues: estrogen-like effects on the vagina, uterus and pituitary of the rat. Endocrinology 91 (1972) 1095-1100.

151. Gellert, R. J. and Heinrichs, W. L. Effects of DDT homologs administered to female rats during the perinatal period. Biol. Neonate 26 (1975) 283-290.

152. Peakall, D. B. p,p'-DDT: effect on calcium metabolism and concentration of estradiol in the blood. Science 168 (1970) 592-594.

153. Alvares, A. P. and Kappas, A. Induction of aryl hydrocarbon hydroxylase by polychlorinated biphenyls in the foeto-placental unit and neonatal livers during lactation. FEBS Letters 50 (1975) 172-174.

154. Holan, G. New halocyclopropane insecticides and the mode of action of DDT. Nature 221 (1969) 1025-1029.

155. Kagan, Y. S., Fudel-Ossipova, S. I., Khaikina, B. J., Kuzminskaya, U. A. and Kouton, S. D. On the problem of the harmful effect of DDT and its mechanism of action. Residue Reviews 27 (1969) 43-79.

156. Carson, R. Silent Spring. Greenwich, Conn.: Fawcett Publications, Inc. 1962.

157. Hickey, J. J. (Ed.) Peregrine Falcon Populations, Their Biology and Decline. Madison, Wisc.: Univ. of Wisc. Press, 1969.

158. U.S. The Executive Office of the President. Office of Science and Technology. Ecological Effects of Pesticides on Nontarget Species by D. Pimentel. Washington, D. C.: Government Printing Office, 1971.

159. Robinson, J. Dynamics of organochlorine insecticides in vertebrates and ecosystems. Nature 215 (1967) 33-35.

160. Risenbrough, R. W., Rieche, P., Herman, S. G., Peakall, D. B., Kirven M. N. Polychlorinated biphenyls in the global ecosystem. Nature 220 (1968) 1098-1102.

161. Ottoboni, A. Effect of DDT on reproduction in the rat. Toxicol. Appl. Pharmacol. 14 (1969) 74-81.

162. Duby, R. T., Travis, H. F. and Terrill, C. E. Uterotropic activity of DDT in rats and mink and its influence on reproduction in the rat. Toxicol. Appl. Pharmacol. 18 (1971) 348-355.

163. Babický, A., Pařízek, J., Oštádalová, I. and Kolář, J. Initial solid food intake and growth of young rats in nests of different sizes. Physiologica Bohemoslovaca 22 (1973) 557-566.

164. Pfeiffer, C. A. Sexual differences of the hypophyses and their determination by the gonads. Am. J. Anat. 58 (1936) 195-225.

165. Preece, A. A Manual for Histologic Technicians. Boston, Mass.: Little, Brown and Co., 1972.

166. Bitman, J., Cecil, H. and Fries, G. F. Nonconversion of o,p'-DDT to p,p'-DDT in rats, sheep, chickens and quail. Science 174 (1971) 64-66.

167. Alltech Associates. Gas Chromatography Supplies, Catalog No. 15, 1974-1975.

168. Bush, E. T. and Hansen, D. L. Improvement of liquid scintillation counting efficiencies by optimization of scintillator composition. Relative efficiencies for three new fluors. In Radioisotope Sample Measurement Techniques in Medicine and Biology, 395-408. Vienna: International Atomic Energy Agency, 1965.

169. Niswender, G. D., Midgley, A. R., Jr., Monroe, S. E. and Reichert, L. E., Jr. Radioimmunoassay for rat luteinizing hormone with antiovine LH serum and ovine-LH-^{131}I. *Proc. Soc. Exp. Biol. Med.* *128* (1968) 395-408.

170. Hutchinson, J. S. and Goldman, B. D. The relationship between the rate of testosterone infusion and gonadotropin secretion. *Endocrinology* *97* (1975) 725-730.

171. Schally, A. V., Arimura, A., Baba, Y., Nair, R. M. G., Matsuo, H., Redding, T. W. and Debeljuk, L. Isolation and properties of the FSH and LH-releasing hormone. *Biochem. Biophys. Res. Commun.* *43* (1971) 393-399.

172. Aiyer, M. S., Chiappa, S. A. and Fink, G. A priming effect of luteinizing hormone releasing factor on the anterior pituitary gland in the female rat. *J. Endocr.* *62* (1974) 573-588.

173. Greeley, G. H., Jr., Allen, M. B. and Mahesh, V. B. FSH and LH response in the rat after intravenous, intracarotid or subcutaneous administration of LH-RH. *Proc. Soc. Exp. Biol. Med.* *147* (1974) 859-862.

174. Mortimer, C. H., Besser, G. M., Hook, J. and McNeilly, A. S. Intravenous, intramuscular, subcutaneous and intranasal administration of LH/FSH-RH: the duration of effect and occurrence of asynchronous pulsatile release of LH and FSH. *Clinical Endocrinology* *3* (1974) 19-25.

175. Harbison, R. D. Comparative toxicity of some selected pesticides in neonatal and adult rats. *Toxicol. Appl. Pharmacol.* *32* (1975) 443-446.

176. Mattingly, D. A simple fluorimetric method for the estimation of free 11-hydroxycorticoids in human plasma. *J. Clin. Pathol.* *15* (1962) 374-379.

177. Silber, R. H., Busch, R. D. and Oslapas, R. Practical procedure for estimation of corticosterone or hydrocortisone. *Clin. Chem.* *4* (1958) 278-285.

178. Discussion, Chapter 20. In *Statistics in Endocrinology*, 279-392. Ed. J. W. McArthur and T. Colton. Cambridge, Mass.: The M.I.T. Press, 1970.

179. Rodbard, D., Raylord, P. L. and Ross, G. T. Statistical quality control of radioimmunoassays, Chapter 23. In Statistics in Endocrinology, 411-429. Ed. J. W. McArthur and T. Colton. Cambridge, Mass.: The M.I.T. Press, 1970.

180. Yamamoto, M., Diebel, N. D., Bogdanove, E. M. Analysis of initial and delayed effects of orchidectomy and ovariectomy on pituitary and serum LH levels in adult and immature rats. Endocrinology 86 (1970) 1102-1111.

181. Midgley, A. R., Jr., Niswender, G. D., Gay, V. L. and Reickert, L. E., Jr. Use of antibodies for characterization of gonadotropins and steroids. Rec. Prog. Hormone Res. 27 (1971) 235-301.

182. Karsch, F. J. personal communication.

183. Dunn, T. D., Arimura, A. and Scheving, L. E. Effect of stress on circadian periodicity in serum LH and prolactin concentration. Endocrinology 90 (1972) 29-33.

184. Hefco, E. and Lackey, R. L. Circadian variations of plasma levels of LH, FSH, prolactin and GH in normal male rats. Fed. Proc. 32 (1973) 281.

185. Krulich, L. and Illner, P. Effect of stress on plasma LH, FSH, prolactin, TSH and GH in normal male rats. Fed. Proc. 32 (1973) 281.

186. Lawton, I. E. and Smith, S. W. LH secretory patterns in intact and gonadectomized male and female rats. Am. J. Physiol. 219 (1970) 1019-1022.

187. Ottoboni, A. and Ferguson, J. I. Excretion of DDT compounds in rat milk. Toxicol. Appl. Pharmacol. 15 (1969) 56-61.

188. Widmark, G., Institute of Analytical Chemistry, Univ. of Stockholm, Sweden. In a letter to J. B. Moore, Vice President and Director of Research, McLaughlin Gormley King Co., Minneapolis, Minnesota. October 26, 1967.

189. McCully, K. A. and McKinley, W. P. Determination of chlorinated pesticide residues in fat by electron capture gas chromatography. J.A.O.A.C. 47 (1964) 652-658.

190. Abou-Donia, M. B. and Menzel, D. B. DDT-type compounds: separation and identification by a system combining thin layer chromatography, gas chromatography, and infrared spectroscopy. *J.A.O.A.C.* *51* (1968) 1247-1260.

191. Moldeus, P. W., Young-nam, C., Cinti, D. L. and Schenkman, J. B. Hepatic organelle interaction-III. Mitochondrial modification of microsomal drug metabolism. *J. Biol. Chem.* *248* (1973) 8574-8584.

192. Coffey, J. C., French, F. S. and Nayfeh, S. N. Metabolism of progesterone by rat testicular homogenates. IV. Further studies of testosterone formation in immature testis *in vitro*. *Endocrinology* *89* (1971) 865-872.

193. Sowell, J. G., Folman, Y. and Eik-Nes, K. B. Androgen metabolism in rat testicular tissue. *Endocrinology* *94* (1974) 346-354.

194. Layne, E. Spectrophotometric and turbidimetric methods for measuring proteins. In *Methods in Enzymology*, *Volume III*, 450-451. Ed. S. P. Colowick and N. O. Kaplan. New York: Academic Press, Inc., 1957.

195. Eik-Nes, K. B. *Gas Phase Chromatography of Steroids*. Monograph on Endocrinology Volume 2. Ed. K. B. Eik-Nes and E. C. Horning. Berlin: Springer-Verlag, 1968.

196. Okita, G. T., Kabara, J. J., Richardson, F. and LeRoy, G. V. Assaying compounds containing ^{3}H and ^{14}C. *Nucleonics* *15(6)* (1957) 111-114.

197. Oštádalová, I. and Pařízek, J. Delayed retardation of somatic growth of rats injected with oestrogens during the first days of postnatal life. *Physiologia Bohemoslovaca* *17* (1968) 217-228.

198. Dorfman, R. I. and Shipley, R. A. *Androgens*. New York: John Wiley and Sons, Inc., 1956.

199. Selye, H. The effect of testosterone on the kidney. *J. Urology* *42* (1939) 637-641.

200. Levine, S. and Mullins, R., Jr., Estrogen administered neonatally affects adult sexual behavior in male and female rats. *Science* *144* (1964) 185-187.

201. Kitay, J. I. Effects of estradiol on pituitary-adrenal function in male and female rats. Endocrinology 72 (1963) 947-954.

202. Simmons, J. E., Di Clementi, D. and Maxted, G. Aspects of adrenal function in neonatally estrogenized male mice. J. Exp. Zool. 186 (1973) 123-126.

203. Freud, J. Conditions of hypertrophy of the seminal vesicles in rats. Biochem. J. 27 (1933) 1438-1450.

204. Yates, F. E., Herbst, A. L. and Urguhart, J. Sex difference in rate of ring A reduction of Δ^4-3-keto-steroids in vitro by rat liver. Endocrinology 63 (1958) 887-902.

205. Sufrin, G. and Coffey, D. S. A comparison of the hormone responsiveness of the prostate and seminal vesicles. Investigative Urology 11 (1974) 386-391.

206. Potter, V. R., Baril, E. F., Watanabe, M. and Whittle, E. D. Systematic oscillations in metabolic functions in liver from rats adapted to controlled feeding schedules. Fed. Proc. 27 (1968) 1238-1245.

207. Schiavi, R. C. Adenohypophyseal and serum gonadotrophins in male rats treated neonatally with estradiol benzoate. Endocrinology 82 (1968) 983-988.

208. Krause, W., Hamm, K. and Weissmuller, J. The effect of DDT on spermatogenesis of the juvenile rat. Bull. Environ. Contam. Toxicol. 14 (1975) 171-179.

209. Maqueo, M. and Kincl, F. A. Testicular histo-morphology of young rats treated with oestradiol-17β-benzoate. Acta Endocrinologica 46 (1964) 25-30.

210. Holmquist, D. L., Retiene, K. and Lipscomb, H. S. Circadian rhythms in rats: effects of random lighting. Science 152 (1966) 662-664.

211. Arai, Y. The content of luteinizing hormone of the anterior pituitary and of luteinizing hormone releasing factor of the hypothalamus in estrogen-induced persistent-diestrus rats. Proc. Japan Acad. 39 (1963) 605-609.

212. Crow, E. L., Davis, F. A. and Maxfield, M. W. Statistics Manual. New York: Dover Publications, Inc., 1960.

213. Croxton, F. E. Elementary Statistics: with Applications in Medicine and the Biological Sciences. New York: Dover Publications, Inc., 1959.

214. Steel, R. G. D. and Torrie, J. H. Principles and Procedures of Statistics, 110-114. New York: McGraw-Hill Book Co., Inc., 1960.

215. Morello, A. Role of DDT-hydroxylation in resistance. Nature 203 (1964) 785-786.

216. Babický, A., Oštádalová, I., Pařizek, J., Kolář, J. and Bíbr, B. Onset and duration of the physiological weaning period for infant rats reared in nests of different sizes. Physiologia Bohemoslovaca 22 (1973) 449-456.

217. Hart, L. G., Schutice, R. W. and Fouts, J. R. Stimulatory effects of chlordane on hepatic microsomal drug metabolism in the rat. Toxicol. Appl. Pharmacol. 5 (1963) 371-386.

218. Cox, W. M., Jr. and Mueller, A. J. The composition of milk from stock rats and an apparatus for milking small laboratory animals. J. Nutrition 13 (1937) 249-261.

219. Burke, J. A. Gas chromatography for pesticide residue analysis; some practical aspects. J.A.O.A.C. 48 (1965) 1037-1058.

220. Hanwell, A. and Linzell, J. L. A simple technique for measuring the rate of milk secretion in the rat. Comp. Biochem. Physiol. 43A (1972) 259-270.

221. Schenkman, J. B., Remmer, H. and Estabrook, R. W. Spectral studies of drug interaction with hepatic microsomal cytochrome. Mol. Pharmacol. 3 (1967) 113-123.

222. Narasimhulu, S., Cooper, D. Y. and Rosenthal, O. Spectrophotometric properties of a Triton-clarified steroid 21-hydroxylase system of adrenocortical microsomes. Life Sciences 4 (1965) 2101-2107.

223. Young, R. B., Bryson, M. J., Sweat, M. L. and Street, J. C. Complexing of DDT and o,p'-DDD with adrenal cytochrome P-450 hydroxylating systems. J. Steroid Biochem. 4 (1973) 585-591.

224. Sandler, R. and Hall, P. F. The response of rat testis to interstitial cell-stimulating hormone in vitro. Comp. Biochem. Physiol. 19 (1966) 833-843.

225. Nayfeh, S. N., Barefoot, S. W., Jr. and Baggett, B. Metabolism of progesterone by rat testicular homogenates. II. Changes with age. Endocrinology 78 (1966) 1041-1048.

226. Inano, H. and Tamaoki, B.-I. Bioconversion of steroids in immature rat testes in vitro. Endocrinology 79 (1966) 579-590.

227. Matsumoto, K. and Yamada, M. 5α-Reduction of testosterone in vitro by rat seminiferous tubules and whole testes at different stages of development. Endocrinology 93 (1973) 253-255.

228. Eik-Nes, K. B. The Androgens of the Testis. New York: Marcel Dekker, Inc., 1970.

229. Sommerville, I. F. Determination of Steroid Hormones. Radiochemical review booklet. Amersham, England: Amersham/Searle Corp., about 1970 (no date given).

230. Joseph, A. A. and Kincl, F. A. Neonatal sterilization of rodents with steroid hormones: 5. A note on the influence of neonatal treatment with estradiol benzoate or testosterone propionate on steroid metabolism in the brain and testes of adult male rats. J. Steroid Biochem. 5 (1974) 227-231.

231. Ettre, L. S. and Slatkis, A. (Ed.) The Practice of Gas Chromatography. New York: Interscience, 1967.

APPENDIX I

THE PROGRAM METFLX

The program METFLX was designed to reduce data generated in a comparative metabolism problem. Its purpose is to work data into a form usable in drawing correlations and conclusions concerning the metabolic flux of a substrate through a tissue when incubated in vitro under a variety of conditions. Specifically, it was written to evaluate a combination of ^{3}H-^{14}C count data and gas chromatographic results which were both to be generated during the course of experiments on androgen production in rat testes under the influence of DDT.

The problem was one of taking recovery data, generated by a ^{14}C internal standard, and combining it with total mass data, generated by gas chromatography, and conversion data, generated by ^{3}H counts derived from labelled substrate, and converting these to: 1) the values which actually occurred within the incubation in terms of total steroid present at given times; 2) the contributions of the ^{3}H substrate, the endogenous substrate and all other substrate sources to the total amount of a particular steroid present; 3) the amount of endogenous steroid present before the start of the experiment; 4) the net converions to and from the substrate and products; and 5) the amounts of a particular steroid which are formed from unlabelled substrates, including the one added, precursors of the one added and all others which generate the product by alternate pathways. Graphs of net conversions at given sampling times and under four different test conditions were plotted to allow graphic comparisons of flux and kinetics.

The calculations for the program were based on those generally accepted for either gas chromatographic or double-label quantitation experiments (195,196,229,231). They were modified to accommodate the simultaneous use of both methods in order to measure flux within a system which contained labelled and unlabelled substrate, endogenous substrate, endogenous substrate precursors, endogenous product, alternate product precursors and an amount of recovery (internal) standard containing a second label.

The flux model, described in Chapter 5, and some initial empirical observations dictated the following equations which yielded the desired pieces of data. Equation 1) calculates the nanomoles of products or substrate found per milligram of protein at any given time under any given condition. This equation was calculated by the function XMOLE.

1) nmoles/mg protein = $(C1/C3) \times (10\ \mathrm{Exp}((\mathrm{Log}_{10}(A1)-SL-AP)/B)) \times (VF/VI) \times FF \times CE \times V \times 0.001\ /GP$

$C1$ = number of ^{14}C counts added

$C3$ = number of ^{14}C counts recovered

Al = chromatographic area of the compound of interest relative to that of the internal standard (chromatographic) in the same injection of sample

SL = the Log_{10} of the ratio of the concentration of the internal standard (chromatographic) in the calibration samples to the concentration of the internal standard in the sample

AP = the intercept of the calibration plot of Log_{10} (relative chromatographic area) versus Log_{10} (compound concentration)

VF = final total volume of the sample

VI = initial total volume of the sample

FF = fraction of total gas flow passing through the capture capillary

CE = efficiency of capillary capture for the compound of interest

V = volume injected into the chromatograph in ul

GP = concentration of protein in the sample incubation in mg/ml

This equation was used to generate the following additional equations and data:

Equation	Data
I	nmoles/mg-protein of substrate in the nonenzymatic incubation
II	nmoles/mg-protein of product in the nonenzymatic incubation

Equation	Data
III	nmoles/mg-protein of substrate at time 0 in a test incubation
IV	nmoles/mg-protein of product at time 0 in a test incubation
V	nmoles/mg-protein of substrate at times greater than 0 in a test incubation
VI	nmoles/mg-protein of product at times greater than 0 in a test incubation

Equation 2) calculated the tritiated product formed during a test incubation. This equation was calculated by function H3 and was referred to as Equation 1 in the output.

2) $\text{nmoles/mg protein} = ((C2 \times (C1/C3))/S0)/GP$

$C2$ = number of tritium counts recovered

$S0$ = the initial specific activity of the added tritiated substrate

Equation 3) calculated the product formed from tritiated and initially present, endogenous substrate during a test incubation. This equation was calculated by function H3E and was referred to as Equation 2 in the output.

3) $\text{nmoles/mg protein} = ((C2 \times C1)/C3)/(C0/((C0/S0) + (III \times GP) - (I))/GP$

$C0$ = initial number of ^3H counts added

Equation 4) calculated product formed from all forms of substrate during a test incubation. This equation was calculated by the function ALL and was referred to as Equation 3 in the output.

4) $\text{nmoles/mg protein} = ((C2 \times C1)/C3/(C0/((C0/S0) + (V \times GP) - (I))/GP$

Finally, from the above equations the following equations and data were obtained:

Equation	Data
X = III-I	nmoles/mg-protein of endogenous substrate
XI = IV-II	nmoles/mg-protein of endogenous product
XII = III-V	the net number of nmoles of substrate converted
XIII = VI-IV	the net number of nmoles of product formed
XIV = VIII-VII	nmoles/mg-protein of product formed from endogenous substrate
XV = IX-VIII	nmoles/mg-protein of product formed from substrate precursors
XVI = VI-IV-IX	nmoles/mg-protein of product formed from sources other than the substrate under study.

Negative values could be obtained from the above calculations. Such values conveyed the information that endogenous product or alternative product precursors had been converted to subsequent products in amounts exceeding the amount of product produced from the specific substrate under study.

Mnemonics for use in METFLX are summarized in Table 10, while the logical sequence of program steps is outlined in the flowchart given in Figure 34.

A printout of the program and its output were generated for exemplary purposes from results collected in a series of control incubations. These incubations were conducted under the conditions described for incubations type 2 and 5 in Table 3 (Chapter 2, Section IV.F.1),i.e., they were incubations of [7 n-^{3}H]-Δ^5-pregnenolone in the presence or absence of the 500 x g supernatants of 6:1 testicular homogenates from adult rats. The homogenates were derived from 4 groups of rats neonatally injected with 5 consecutive daily s.c. injections on days 1-5 of age. The injections were each of 0.05 ml of sesame oil containing: nothing (treatment 1), 40 ug EV (treatment 2), 200 ug TP (treatment 3) or 500 ug o,p'-DDT (treatment 4). After the incubations the steroids were extracted under the protocol listed in Figure 11 (Chapter 2, Section IV.F.1) and were analyzed under the conditions given in Figure 12. The steroids were numbered in order of elution from the GC column: 1 = dehydroepiandrosterone, 2 = Δ^4-androstenedione and testosterone, 3 = Δ^5-pregnenolone, 4 = progesterone and 5 = 17α -hydroxyprogesterone. All the steroids were computed as products in the present program run in order to allow a check on the rate of substrate (product 3) disappearance with time of incubation as calculated by function H3.

Table 10

Mnemonics of METFLX

All Data and calculations were stored in arrays according to incubation type and time point (0, 5, 10, 30 and 60 minutes). Product data and calculations had the added dimension of product number.

Symbol	Definition
ESDATA	Array in which substrate data from the non-enzymatic incubation were stored
ESUB	Vector in which the calculated nmoles/mg-protein of substrate from the nonenzymatic incubation were stored
SDATA	Array in which the substrate data from the enzymatic incubation were stored
SUB	Array in which the calculated nmoles/mg-protein of substrate in the enzymatic incubation were stored
BP	The slope of the standard - calibration - curve of Log$_{10}$(Al) versus Log $_{10}$(concentration)
AP	The intercept of the standard - calibration - curve of Log$_{10}$(Al) versus Log$_{10}$(concentration)
PDATA	Array in which the product data from the non-enzymatic incubation were stored
EPRO	Array in which the calculated nmoles/mg-protein of product in the nonenzymatic incubation were stored
PRO	Array in which the data for the product from the enzymatic incubation were stored
PROD	Array in which the calculated nmoles/mg-protein of product in the enzymatic incubation were stored
XPROD	Array where the calculated values for H3 were stored
YPROD	Array where the calculated values for H3E were stored

Table 10 (Cont.)

Symbol	Definition
ZPROD	Array where the calculated values for ALL were stored
X to XVI	Arrays in which the calculated values for equations X to XVI were stored
T	Vector in which the sampling times were stored
NSCALE	Vector which held scaling information for use in PLOT1
CHAR	Vector which held the character labels for use in PLOT 4
POINT	The value of the ordinate for points plotted by PLOT1-4
GP	Vector which held the values of mg-protein per ml for all the incubation conditions
VI	Vector which held the values of the initial sample volumes for all the incubation conditions
CE	Vector which held the values of the capture efficiency of capillary capture for each of the chromatographic peaks examined
FF	The value which defined the flow fraction of gas which passed through the capillary capture tube
SL	The value which defined the Log$_{10}$ of the ratio of the concentration of the internal chromatographic standard in the calibration mixture over the concentration of the internal chromatographic standard in the measured sample
VF	The value which defined the final total volume of the sample
N	The value which defined the number of products measured
B	BP for the substrate
SO	Initial specific activity of the added tritiated substrate in dpm/nmole
CO	Initial number of tritium counts added in dpm

Table 10 (Cont.)

Symbol	Definition
XMOLE	The function which calculated the total number of nmoles/mg-protein in the sample
PROCAL	The subroutine which calculated PROD, XPROD, YPROD, ZPROD, XI, XIII, XIV, XV and XVI
C1	The initial number of carbon-14 dpm added
C2	The number of tritium dpm recovered
C3	The number of carbon-14 dpm recovered
A1	The area of the chromatographic peak which corresponded to a given compound relative to the area of the internal standard peak in the same injection
V	The volume of the sample injected for measurement
YMIN	The minimum value for the ordinate for a given data plot, used in PLOT1-4
YMAX	The maximum value for the ordinate of a given data plot, used in PLOT1-4
PLOT1-4	A series of computer printer plot subroutines located within the *LIBRARY of the MTS computer system of The University of Michigan
H3	The function which calculated the conversion of tritium dpm from substrate to product for only tritiated substrate
H3E	The function which calculated the conversion of endogenous plus tritiated substrate to product
ALL	The function which calculated the formation of product from all sources of substrate
ALOG10	The single precision estimate of the logarithm to the base 10
SUBO	SUB at time zero

Figure 34

Flowchart for METFLX

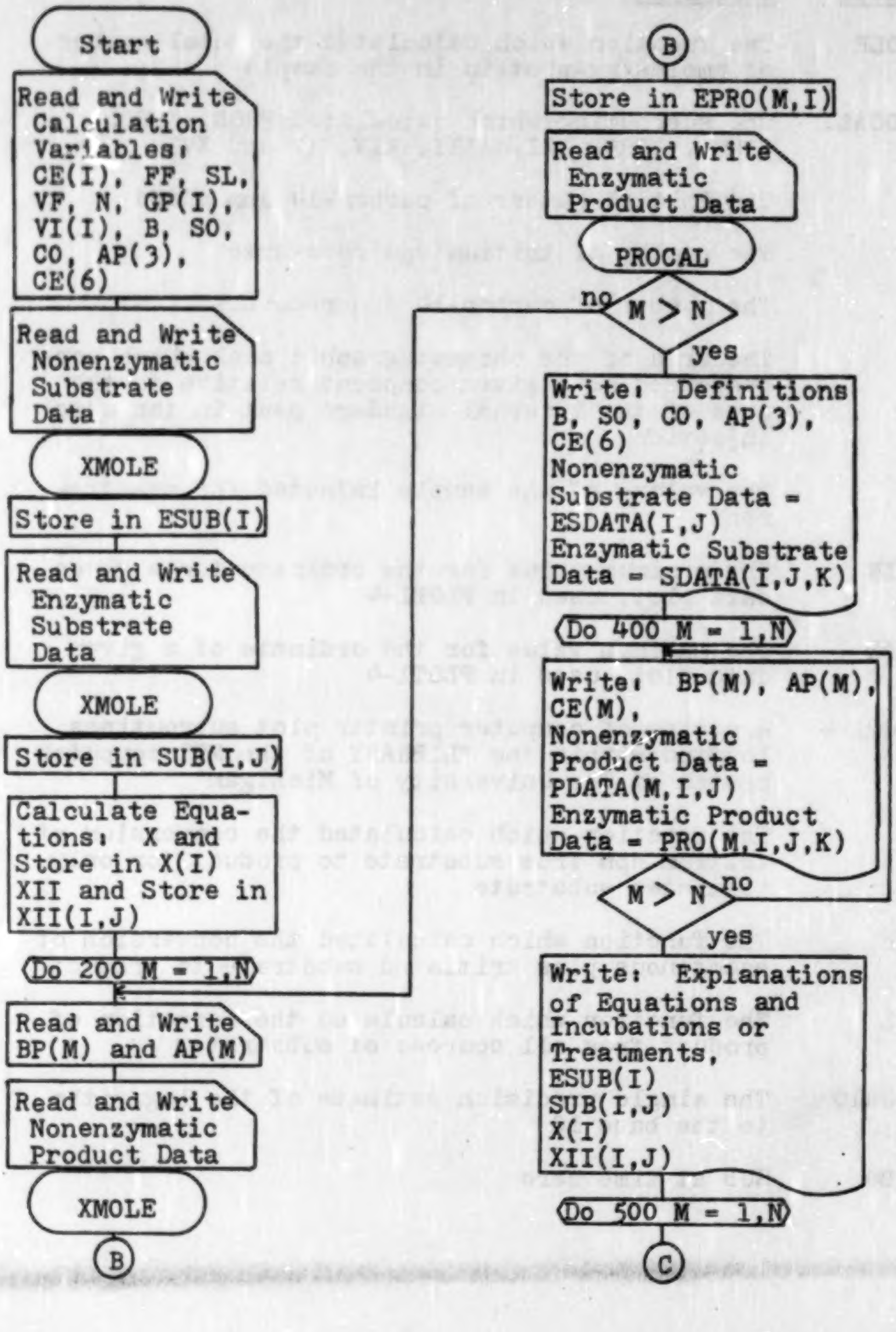
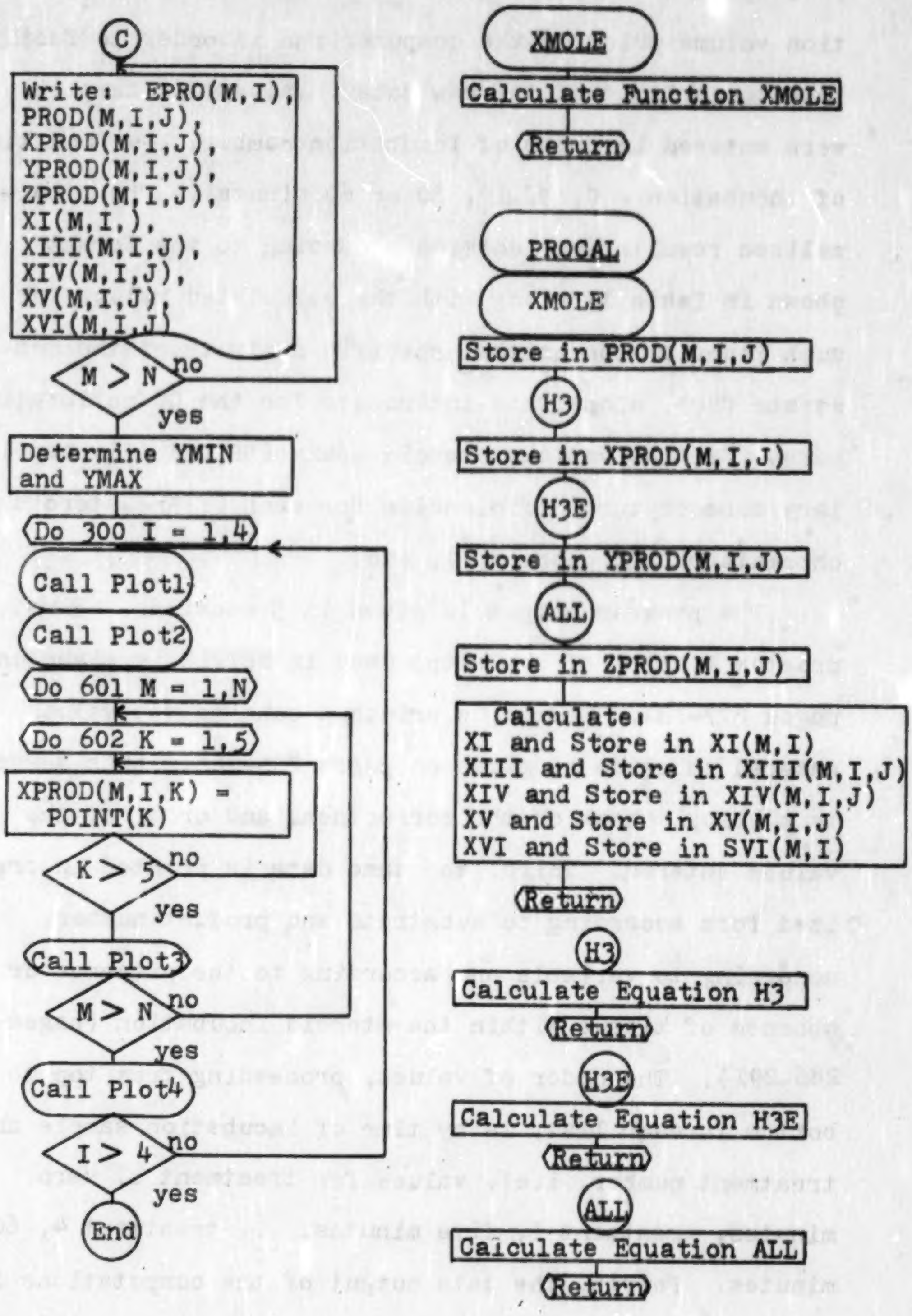

Start
Read and Write Calculation Variables: CE(I), FF, SL, VF, N, GP(I), VI(I), B, SO, CO, AP(3), CE(6)
Read and Write Nonenzymatic Substrate Data
XMOLE
Store in ESUB(I)
Read and Write Enzymatic Substrate Data
XMOLE
Store in SUB(I,J)
Calculate Equations: X and Store in X(I) XII and Store in XII(I,J)
Do 200 M = 1,N
Read and Write BP(M) and AP(M)
Read and Write Nonenzymatic Product Data
XMOLE
B
B
Store in EPRO(M,I)
Read and Write Enzymatic Product Data
PROCAL
no
M > N
yes
Write: Definitions B, SO, CO, AP(3), CE(6) Nonenzymatic Substrate Data = ESDATA(I,J) Enzymatic Substrate Data = SDATA(I,J,K)
Do 400 M = 1,N
Write: BP(M), AP(M), CE(M), Nonenzymatic Product Data = PDATA(M,I,J) Enzymatic Product Data = PRO(M,I,J,K)
M > N
no
yes
Write: Explanations of Equations and Incubations or Treatments, ESUB(I) SUB(I,J) X(I) XII(I,J)
Do 500 M = 1,N
C

Figure 34 (Cont.)


C
Write: EPRO(M,I),
PROD(M,I,J),
XPROD(M,I,J),
YPROD(M,I,J),
ZPROD(M,I,J),
XI(M,I,),
XIII(M,I,J),
XIV(M,I,J),
XV(M,I,J),
XVI(M,I,J)
M > N
no
yes
Determine YMIN and YMAX
Do 300 I = 1,4
Call Plot1
Call Plot2
Do 601 M = 1,N
Do 602 K = 1,5
XPROD(M,I,K) = POINT(K)
K > 5
no
yes
Call Plot3
M > N
no
yes
Call Plot4
I > 4
no
yes
End
XMOLE
Calculate Function XMOLE
Return
PROCAL
XMOLE
Store in PROD(M,I,J)
H3
Store in XPROD(M,I,J)
H3E
Store in YPROD(M,I,J)
ALL
Store in ZPROD(M,I,J)
Calculate:
XI and Store in XI(M,I)
XIII and Store in XIII(M,I,J)
XIV and Store in XIV(M,I,J)
XV and Store in XV(M,I,J)
XVI and Store in SVI(M,I)
Return
H3
Calculate Equation H3
Return
H3E
Calculate Equation H3E
Return
ALL
Calculate Equation ALL
Return

Chromatographic area data and radioactivity levels (^{14}C-dpm and ^{3}H dpm) were normalized to 1 ul of GC injection volume prior to the computer run in order to facilitate comparisons of the raw data. Values for samples were entered in order of incubation number, 1-4, and time of incubation - 0, 5, 10, 30 or 60 minutes. These normalized results were entered according to the format shown in Table 11 along with the calculated values for such items as the initial specific activity of the substrate (SO), slopes and intercepts for the GC calibration curves for the various steroid peaks (BP and AP), capillary tube capture efficiencies for each of the steroid chromatographic peaks (CE), etc.

The program output is given in 5 sections. First, a program listing of the steps used in METFLX is given on pages 277-282. Second, a printout echoing the values entered on cards is given on pages 283-285; this serves as a visual check on the correctness and order of the values entered. Third, the same data is printed in organized form according to substrate and product number, according to variable and according to the presence or absence of tissue within the steroid incubation (pages 286-291). The order of values, proceeding from top to bottom in each list, is by time of incubation sample and treatment number, i.e., values for treatment 1, zero minutes, treatment 1, five minutes, ... treatment 4, 60 minutes. Fourth, the data output of the computations is

Table 11

Description of the Input Data Format for METFLX

There are 10 types of data cards which were entered in the following order:

1. One card
   - Constants CE(1), CE(2), ... , CE(6)
2. One card
   - Constants FF, SL, VF
3. One card
   - Constants N, GP(1), GP(2), GP(3), GP(4)
4. One card
   - Constants VI(1), VI(2), VI(3) and VI(4)
5. One card
   - Constants B, SO, CO, AP(3), CE(6)
6. Five cards
   - Data cards for the substrate from the enzymatic incubation
   - Each card contained 4 values: C1, C3, A1 and V
   - Cards were read according to increasing time: 0, 5, 10, 30, 60 minutes
7. Twenty cards
   - Data cards for the substrate from the enzymatic incubations
   - Each card contained values for: C1, C3, A1 and V
   - Each card represented one time point; they were read in the same order as in 6.
   - Each set of 5 cards represented one incubation; there were a total of 4 incubations
8. One card
   - Constants BP and AP
9. Five cards
   - Data cards for the product from the nonenzymatic incubation
   - Each card contained 4 values: C1, C3, A1 and V
   - Cards were read according to increasing time: 0, 5, 10, 30 and 60 minutes

Table 11 (Cont.)

10. Twenty cards
    Data cards for the product from the
    enzymatic incubation
    Each card contained 5 values: C1, C2, C3,
    A1 and V
    Each card represented one time point; they
    were read in the same order as in 9.
    Each set of 5 cards represented one incubation; there were a total of 4 incubations

Formats 8., 9. and 10. were repeated as many times as there were products.

printed (pages 292 to 302). The substrate is listed first followed by each of the products (product 3 = substrate). The values for the various incubation conditions, times and equations are printed in clearly labelled order. Fifth, plots of the content of ^{3}H-labelled substrate and products versus time of incubation are given for each animal treatment group. These allow easy comparison of rates and levels of conversion[1].

The results generated demonstrate the need for several modifications in the experimental procedures. First, the total nanomoles of substrate and products closely resemble the amounts added as carrier. This destroys the utility of the program for computing endogenous levels of substrate and/or product or quantities based on those levels. Therefore, large reductions, or elimination of carrier is suggested. This might necessitate the use of electron capture detection of appropriate steroid derivatives but that possibility is presently used in a number of laboratories (195;229). Second, the need to use uniform sample sizes is illustrated by the differences seen between treatment groups 1-3, which used 1 ml sampling aliquots and those in group 4 which used 5 ml sampling aliquots.

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[1] In the program printout the following numbers and letters are used to identify specific steroid chromatographic peaks: 1=A=dehydroepiandrosterone, 2=B=Δ^4-androstenedione + testosterone, 3=C=Δ^5-pregnenolone, 4=D=progesterone, 5=E=17α-hydroxyprogesterone.

Modifications of the program are indeed possible. Extensions of printout to include plots of all the equation results and inclusion of the protein concentrations in each incubation could easily be made. Similarly, additional correction or dilution factors may be included as well as extensions to different numbers of sampling times or incubation or treatment conditions.

Finally, utility of the program is not limited to the coupled GC-scintillation counting system described here. Any mass determination which does not interfere with the simultaneous quantitation of ^{3}H- and ^{14}C- may just as easily be used in the context of this program. Similarly, the program is not limited to use with steroids but is easily extended to numerous other metabolic problems. It is hoped that the generality of its inception may serve to make the program useful in many studies both in and out of the area of steroid biochemistry.

```
C METFLX CALCULATES THE METABOLIC FLUX OF COMPOUNDS IN A TISSUE
C INCUBATION BASED ON DATA GENERATED BY GC AND DOUBLE-LABEL SCINTILLA-
C TION COUNTING.  INITIALLY APPLIED TO ANDROGEN METABOLISM.
      DIMENSION ESDATA(5,5),ESUB(5),SDATA(4,5,5),SUB(4,5),BP(6),AP(6)
      DIMENSION PDATA(6,5,5),EPRO(6,5),PRO(6,4,5,6)
      DIMENSION PROD(6,4,5),XPROD(6,4,5),YPROD(6,4,5),ZPROD(6,4,5)
      DIMENSION X(4),XI(6,4),XII(4,5),XIII(6,4,5),XIV(6,4,5),
     1XV(6,4,5),XVI(6,4,5)
      DIMENSION T(5),NSCALE(5),CHAR(7)
      DIMENSION POINT(5),GP(4),VI(4)
      DIMENSION CE(6)
  410 FORMAT(6F10.8)
      READ(5,410)(CE(I),I=1,6)
      WRITE(6,411)(CE(I),I=1,6)
  411 FORMAT(' ',6F10.8)
      READ(5,420)FF,SL,VF
      WRITE(6,421)FF,SL,VF
  420 FORMAT(3F10.8)
  421 FORMAT(' ',2F10.8,F10.3)
      READ(5,201)N,(GP(I),I=1,4)
      WRITE(6,202)N,(GP(I),I=1,4)
  201 FORMAT(I1,4F10.8)
  202 FORMAT(' ',I3,4F10.4)
      READ(5,207)(VI(I),I=1,4)
  207 FORMAT(4F10.3)
      WRITE(6,208)(VI(I),I=1,4)
  208 FORMAT(' ',4F10.3)
      READ(5,1)B,SO,CO,AP(3),CE(6)
      WRITE(6,203)B,SO,CO,AP(3),CE(6)
  1   FORMAT(5F10.8)
  203 FORMAT(' ',F10.6,2F10.2,F10.6,F10.8)
      READ(5,2)((ESDATA(I,J),J=1,4),I=1,5)
      WRITE(6,204)((ESDATA(K,L),L=1,4),K=1,5)
    2 FORMAT(F10.4,10X,3F10.4)
  204 FORMAT(' ',F10.2,10X,F10.2,F10.4,F10.2)
      DO 100 I=1,5
  100 ESUB(I)=XMOLE(ESDATA(I,1),ESDATA(I,2),ESDATA(I,3),ESDATA(I,4),
     1B,1.0,AP(3),FF,SL,CE(6),VI(1),VF)
      READ(5,2)(((SDATA(I,J,K),K=1,4),J=1,5),I=1,4)
      WRITE(6,204)(((SDATA(L,M,K),K=1,4),M=1,5),L=1,4)
      DO 101 I=1,4
      DO 101 J=1,5
  101 SUB(I,J)=XMOLE(SDATA(I,J,1),SDATA(I,J,2),SDATA(I,J,3),
     3SDATA(I,J,4),B,GP(I),AP(3),FF,SL,CE(6),VI(I),VF)
      DO 105 I=1,4
  105 X(I)=SUB(I,1)-ESUB(I)/GP(I)
      DO 106 I=1,4
      DO 106 J=1,5
  106 XII(I,J)=SUB(I,1)-SUB(I,J)
      DO 200 M=1,N
      READ(5,23)BP(M),AP(M)
      WRITE(6,205)BP(M),AP(M)
   23 FORMAT(2F10.6)
  205 FORMAT(' ',2F10.6)
```

```
      READ(5,2)((PDATA(M,I,J),J=1,4),I=1,5)
      WRITE(6,204)((PDATA(M,K,L),L=1,4),K=1,5)
      DO 102 I=1,5
  102 EPRO(M,I)=XMOLE(PDATA(M,I,1),PDATA(M,I,2),PDATA(M,I,3),
     5PDATA(M,I,4),BP(M),1.0,AP(M),FF,SL,CE(M),VI(1),VF)
      READ(5,3)(((PRO(M,I,J,K),K=1,5),J=1,5),I=1,4)
      WRITE(6,206)(((PRO(M,I,J,K),K=1,5),J=1,5),I=1,4)
    3 FORMAT(3F10.2,F10.4,F10.5)
  206 FORMAT(3F10.2,F10.4,F10.2)
      CALL PROCAL(PRO,SUB,ESUB,EPRO,BP,SO,CO,PROD,XPROD,YPROD,ZPROD,
     4XI,XIII,XIV,XV,XVI,GP,M,AP,FF,SL,CE,VI,VF)
  200 CONTINUE
      WRITE(6,660)
  660 FORMAT('1','DEFINITION OF VARIABLES:')
      WRITE(6,670)
  670 FORMAT('0',4X,'B(P) - SLOPE OF STANDARD PLOT OF RELATIVE AREA VERS
     1US CONCENTRATION OF X')
      WRITE(6,881)
  881 FORMAT(' ',4X,'AP - INTERCEPT OF STANDARD PLOT OF RELATIVE AREA VE
     3RSUS CONCENTRATION OF X')
      WRITE(6,680)
  680 FORMAT(' ',4X,'SO - INITIAL SPECIFIC ACTIVITY, DPM/NM, OF 3H SUBST
     1RATE ADDED')
      WRITE(6,690)
  690 FORMAT(' ',4X,'CO - INITIAL NUMBER OF 3H-SUBSTRATE COUNTS ADDED TO
     1 SAMPLE')
      WRITE(6,700)
  700 FORMAT(' ',4X,'C1 - INITIAL NUMBER OF 14C DPM ADDED TO SAMPLE AS X
     1')
      WRITE(6,710)
  710 FORMAT(' ',4X,'C2 - NUMBER OF 3H DPM RECOVERED FROM SAMPLE AS X')
      WRITE(6,720)
  720 FORMAT(' ',4X,'C3 - NUMBER OF 14C DPM RECOVERED FROM SAMPLE AS X')
      WRITE(6,730)
  730 FORMAT(' ',4X,'A1 - RELATIVE AREA OF PEAK FROM COMPONENT X IN ALIQ
     1UOT OF SAMPLE')
      WRITE(6,750)
  750 FORMAT(' ',4X,'V - VOLUME INJECTED FOR MEASUREMENT')
      WRITE(6,880)
  880 FORMAT(' ',4X,'CE - EFFICIENCY OF CAPILLARY CAPTURE FOR THIS COMPONENT
      WRITE(6,61)B,SO,CO,AP(3),CE(6)
   61 FORMAT('-','B=',F10.6,3X,'SO=',F10.2,3X,'CO=',F10.2,3X,'AP=',F10.6 'CE
     3=',F8.6)
      WRITE(6,62)
   62 FORMAT('-','NONENZYMATIC SUBSTRATE DATA')
      WRITE(6,63)
   63 FORMAT('-',4X,'C1',10X,'C3',9X,'A1',9X,'V')
      WRITE(6,64)((ESDATA(I,J),J=1,4),I=1,5)
   64 FORMAT(' ',F10.2,F10.2,F10.4,F10.2)
      WRITE(6,65)
   65 FORMAT('-','ENZYMATIC SUBSTRATE DATA')
      WRITE(6,63)
      WRITE(6,64)(((SDATA(I,J,K),K=1,4),J=1,5),I=1,4)
      DO 400 M=1,N
      WRITE(6,69)BP(M),AP(M),CE(M)
   69 FORMAT('-','BP=',F10.6,5X,'AP=',F10.6,5X,'CE=',F10.6)
      L=M
      WRITE(6,66)L
   66 FORMAT('-','NONENZYMATIC PRODUCT',1X,I1,1X,'DATA')
      WRITE(6,63)
      WRITE(6,64)((PDATA(M,I,J),J=1,4),I=1,5)
      WRITE(6,67)L
   67 FORMAT('-','ENZYMATIC PRODUCT',1X,I1,1X,'DATA')
      WRITE(6,68)
   68 FORMAT(4X,'C1',10X,'C2',8X,'C3',8X,'A1',9X,'V')
      WRITE(6,70)(((PRO(M,I,J,K),K=1,5),J=1,5),I=1,4)
   70 FORMAT(' ',3F10.2,F10.4,F10.2)
  400 CONTINUE
      WRITE(6,40)
   40 FORMAT('1','EQUATIONS:')
      WRITE(6,41)
   41 FORMAT('0',4X,'1 - 3H PRODUCT FORMED')
      WRITE(6,42)
   42 FORMAT(' ',4X,'2 - PRODUCT FORMED FROM 3H AND ENDOGENOUS SUBSTRATE')
      WRITE(6,43)
   43 FORMAT(' ',4X,'3 - PRODUCT FORMED FROM ALL SOURCES')
      WRITE(6,44)
   44 FORMAT(' ',4X,'X - ENDOGENOUS SUBSTRATE')
      WRITE(6,45)
   45 FORMAT(' ',4X,'XI - ENDOGENOUS PRODUCT')
      WRITE(6,46)
   46 FORMAT(' ',4X,'XII - NET CONVERSION OF SUBSTRATE')
      WRITE(6,47)
   47 FORMAT(' ',4X,'XIII - NET FORMATION OF PRODUCT')
      WRITE(6,48)
   48 FORMAT(' ',4X,'XIV - PRODUCT FORMED FROM ENDOGENOUS SUBSTRATE')
      WRITE(6,49)
   49 FORMAT (' ',4X,'XV - PRODUCT FORMED FROM SUBSTRATE PRECURSORS')
      WRITE(6,50)
   50 FORMAT(' ',4X,'XVI - PRODUCT FORMED FROM OTHER SUBSTRATES')
      WRITE(6,800)
  800 FORMAT('-','ANIMAL TREATMENTS:')
      WRITE(6,810)
  810 FORMAT('0',4X,'1(I) - CONTROL, SESAME OIL INJECTED')
      WRITE(6,820)
  820 FORMAT(' ',4X,'2(II) - ESTRADIOL INJECTED')
      WRITE (6,830)
  830 FORMAT(' ',4X,'3(III) - TESTOSTERONE INJECTED')
      WRITE(6,840)
  840 FORMAT(' ',4X,'4(IV) - DDT INJECTED')
      WRITE(6,4)
    4 FORMAT('-','NMOLES SUBSTRATE: NONENZYMATIC INCUBATION SAMPLE')
      WRITE(6,5)
    5 FORMAT('0',8X,'0 MIN',5X,'5 MIN',4X,'10 MIN',4X,'30 MIN',4X,
     2'60 MIN')
      WRITE(6,6)(ESUB(I),I=1,5)
    6 FORMAT(' ',4X,5(F10.4))
      WRITE(6,7)
    7 FORMAT('-','NMOLES/MG-PROTEIN, SUBSTRATE: ENZYMATIC INCUBATION')
      WRITE(6,5)
      WRITE(6,8)(I,(SUB(I,J),J=1,5),I=1,4)
    8 FORMAT(' ',I1,3X,5F10.4)
      WRITE(6,15)
   15 FORMAT('-','EQUATION X')
      WRITE(6,16)(I,X(I),I=1,4)
   16 FORMAT(' ',I1,3X,F10.4)
      WRITE(6,18)
   18 FORMAT('-','EQUATION XII')
      WRITE(6,5)
      WRITE(6,8)(I,(XII(I,J),J=1,5),I=1,4)
      DO 500 M=1,N
      L=M
      WRITE(6,31)L
   31 FORMAT('1',21X,'PRODUCT',1X,I1)
      WRITE(6,9)
    9 FORMAT('-','NMOLES PRODUCT: NONENZYMATIC INCUBATION SAMPLE')
      WRITE(6,5)
      WRITE(6,6)(EPRO(M,I),I=1,5)
      WRITE(6,10)
   10 FORMAT('-','NMOLES/MG-PROTEIN, PRODUCT: ENZYMATIC INCUBATION')
      WRITE(6,5)
      WRITE(6,8)(I,(PROD(M,I,J),J=1,5),I=1,4)
      WRITE(6,12)
   12 FORMAT('-','EQUATION 1')
      WRITE(6,5)
      WRITE(6,8)(I,(XPROD(M,I,J),J=1,5),I=1,4)
      WRITE(6,13)
   13 FORMAT('-','EQUATION 2')
      WRITE(6,5)
      WRITE(6,8)(I,(YPROD(M,I,J),J=1,5),I=1,4)
      WRITE(6,14)
   14 FORMAT('-','EQUATION 3')
      WRITE(6,5)
      WRITE(6,8)(I,(ZPROD(M,I,J),J=1,5),I=1,4)
      WRITE(6,17)
   17 FORMAT('-','EQUATION XI')
      WRITE(6,16)(I,XI(M,I),I=1,4)
      WRITE(6,19)
   19 FORMAT('-','EQUATION XIII')
      WRITE(6,5)
      WRITE(6,8)(I,(XIII(M,I,J),J=1,5),I=1,4)
      WRITE(6,20)
   20 FORMAT('-','EQUATION XIV')
      WRITE(6,5)
      WRITE(6,8)(I,(XIV(M,I,J),J=1,5),I=1,4)
      WRITE(6,21)
   21 FORMAT('-','EQUATION XV')
      WRITE(6,5)
      WRITE(6,8)(I,(XV(M,I,J),J=1,5),I=1,4)
      WRITE(6,22)
   22 FORMAT('-','EQUATION XVI')
      WRITE(6,5)
      WRITE(6,8)(I,(XVI(M,I,J),J=1,5),I=1,4)
  500 CONTINUE
      T(1)=0.
      T(2)=5.
      T(3)=10.
      T(4)=30.
      T(5)=60.
      DATA CHAR(1),CHAR(2),CHAR(3),CHAR(4),CHAR(5),CHAR(6)/'A','B',
     4'C','D','E','F'/
      YMAX=0
      YMIN=0
      DO 900 M=1,N
      DO 900 I=1,4
      DO 900 J=1,5
      IF(XPROD(M,I,J).GT.YMAX) GO TO 901
      IF(XPROD(M,I,J).LT.YMIN) GO TO 902
      GO TO 903
  902 YMIN=XPROD(M,I,J)
      GO TO 903
  901 YMAX=XPROD(M,I,J)
  903 CONTINUE
  900 CONTINUE
      DO 300 I=1,4
      CALL PLOT1(0,6,9,7,9)
      CALL PLOT2(0,T(5),T(1),YMAX,YMIN)
      DO 601 M=1,N
      DO 602 K=1,5
  602 POINT(K)=XPROD(M,I,K)
  601 CALL PLOT3(CHAR(M),T,POINT,5,4)
      GO TO(81,82,83,84),I
   81 WRITE(6,85)
   85 FORMAT('1',26X,'CONVERSION OF 3H: TREATMENT I')
      GO TO 89
   82 WRITE(6,86)
   86 FORMAT('1',26X,'CONVERSION OF 3H: TREATMENT II')
      GO TO 89
   83 WRITE(6,87)
   87 FORMAT('1',26X,'CONVERSION OF 3H: TREATMENT III')
      GO TO 89
   84 WRITE(6,88)
   88 FORMAT('1',26X,'CONVERSION OF 3H: TREATMENT IV')
   89 CALL PLOT4(17,'NMOLES/MG PROTEIN')
  300 WRITE(6,303)
  303 FORMAT(' ',34X,'TIME IN MINUTES')
      STOP
      END
      SUBROUTINE PROCAL(PRO,SUB,ESUB,EPRO,BP,SO,CO,PROD,XPROD,YPROD,
     5ZPROD,XI,XIII,XIV,XV,XVI,GP,M,AP,PF,SL,CE,VI,VF)
      DIMENSION PRO(6,4,5,6),SUB(4,5),ESUB(5),EPRO(6,5)
      DIMENSION PROD(6,4,5),XPROD(6,4,5),YPROD(6,4,5),ZPROD(6,4,5)
      DIMENSION XI(6,4),XIII(6,4,5),XIV(6,4,5),BP(6)
      DIMENSION XV(6,4,5),XVI(6,4,5),GP(4),VI(4)
      DIMENSION AP(6),CE(6)
      DO 99 I=1,4
   DO 99 J=1,5
   PROD(M,I,J.=XMOLE(PRO(M,I,J,1),PRO(M,I,J,3),PRO(M,I,J,4),
  7PRO(M,I,J,5),BP(M),GP(I),AP(M),FF,SL,CE(M),VI(I),VF)
   XPROD(M,I,J)=H3(PRO(M,I,J,1),PRO(M,I,J,2),PRO(M,I,J,3),SO,GP(I))
   YPROD(M,I,J)=H3E(CO,PRO(M,I,J,1),PRO(M,I,J,2),PRO(M,I,J,3),SO,
  8SUB(I,1),ESUB(J),GP(I))
99 ZPROD(M,I,J)=ALL(CO,PRO(M,I,J,1),PRO(M,I,J,2),PRO(M,I,J,3),SO,
  9SUB(I,J),ESUB(J),GP(I))
   DO 98 I=1,4
98 XI(M,I)=PROD(M,I,1)-EPRO(M,1)/GP(I)
   DO 97 I=1,4
   DO 97 J=1,5
97 XIII(M,I,J)=PROD(M,I,J)-PROD(M,I,1)
   DO 96 I=1,4
   DO 96 J=1,5
   XIV(M,I,J)=YPROD(M,I,J)-PROD(M,I,1)
   XV(M,I,J)=ZPROD(M,I,J)-YPROD(M,I,J)
96 XVI(M,I,J)=XIII(M,I,J)-ZPROD(M,I,J)
   RETURN
   END
   FUNCTION XMOLE(C1,C3,A1,V,B,GP,AP,FF,SL,CE,VI,VF)
   XMOLE=((C1/C3)*(10**(((ALOG10(A..)-SL-AP)/B))*(VF/VI)*FF*CE*V*.001)/GP
   RETURN
   END
   FUNCTION H3(C1,C2,C3,SO,GP)
   H3=((C2*(C1/C3))/SO)/GP
   RETURN
   END
   FUNCTION H3E(CO,C1,C2,C3,SO,SUBO,ESUB,GP)
   H3E=((C2*(C1/C3))/(CO/((CO/SO)+(SUBO*GP)-(ESUB))))/GP
   RETURN
   END
   FUNCTION ALL(CO,C1,C2,C3,SO,SUB,ESUB,GP)
   ALL=((C2*(C1/C3))/(CO/((CO/SO)+(SUB*GP)-(ESUB))))/G.
   RETURN
   END
```

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0.685827970.589727340.884790600.780579860.491454540.88479060
0.74367738-.25467902     0.200
  5   9.1730    7.7820    7.7030  29.4550
     1.000     1.000     1.000     5.000
  1.0469241898380.001846948.00 -3.5120810.88479060
  62016.00               90.88    0.8440      1.00
  62016.00               90.88    0.8440      1.00
  62016.00               90.88    0.8440      1.00
  62016.00               93.60    0.7870      1.00
  62016.00               93.60    0.7870      1.00
  62016.00               33.93    0.4520      1.00
  62016.00               80.09    0.5480      1.00
  62016.00               74.55    0.5210      1.00
  62016.00               76.88    0.8005      1.00
  62016.00               39.62    0.6965      1.00
  62016.00               51.21    0.7480      1.00
  62016.00               90.99    0.7880      1.00
  62016.00               92.45    0.7345      1.00
  62016.00               92.75    0.7635      1.00
  62016.00               45.64    0.8580      1.00
  62016.00               54.05    0.8295      1.00
  62016.00               95.06    0.9090      1.00
  62016.00               83.07    0.7530      1.00
  62016.00               91.89    0.8105      1.00
  62016.00               49.49    0.6805      1.00
  62016.00               53.78    0.5190      1.00
  62016.00               50.78    0.3375      1.00
  62016.00               61.51    0.3970      1.00
  62016.00               48.10    0.3240      1.00
  62016.00               37.27    0.3840      1.00
  1.005030 -3.375531
  61734.00               76.27    0.8280      1.00
  61734.00               76.27    0.8280      1.00
  61734.00               76.27    0.8280      1.00
  61734.00               79.78    0.7940      1.00
  61734.00               79.78    0.7940      1.00
61734.00      14.52     22.86    0.4105       1.00
61734.00      73.67     75.35    0.4670       1.00
61734.00      94.48     64.60    0.4235       1.00
61734.00     151.77     80.27    0.7795       1.00
61734.00     144.60     26.40    0.5770       1.00
61734.00      18.45     32.73    0.6320       1.00
61734.00     495.79     87.27    0.7210       1.00
61734.00     224.79     72.11    0.6375       1.00
61734.00     435.25     83.53    0.7075       1.00
61734.00     236.25     29.53    0.7395       1.00
61734.00      41.51     50.51    0.7130       1.00
61734.00     155.66     96.96    0.7325       1.00
61734.00      83.03     78.33    0.7210       1.00
61734.00     270.20     88.96    0.8045       1.00
61734.00     249.77     42.66    0.5830       1.00
61734.00     153.70     37.56    0.4960       1.00
61734.00     194.15     50.26    0.3860       1.00
61734.00     361.49     53.23    0.4340       1.00
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   61734.00     631.14      43.67    0.3850        1.00
   61734.00     531.94      30.51    0.3315        1.00
    0.988246 -3.357901
    128091.00               163.41    1.8700        1.00
    128091.00               163.41    1.8700        1.00
    128091.00               163.41    1.8700        1.00
    128091.00               163.38    1.6890        1.00
    128091.00               163.38    1.6890        1.00
   128091.00      35.54      54.86    0.9385       1.00
   128091.00      58.90     150.90    1.2230       1.00
   128091.00     158.72     127.57    1.0495       1.00
   128091.00    1396.45     160.67    1.5425       1.00
   128091.00     635.75      60.34    1.2985       1.00
   128091.00      42.07      65.77    1.3160       1.00
   128091.00     274.97     173.57    1.4615       1.00
   128091.00    1445.56     184.33    1.6740       1.00
   128091.00    1334.55     169.50    1.5400       1.00
   128091.00     655.33      70.31    1.5250       1.00
   128091.00      48.09      93.40    1.2285       1.00
   128091.00     131.50     182.33    1.5965       1.00
   128091.00     453.02     169.02    1.6350       1.00
   128091.00    1508.75     183.81    1.6060       1.00
   128091.00     876.23      95.52    1.3680       1.00
   128091.00     268.61      99.22    1.1020       1.00
   128091.00     208.53     109.81    0.8220       1.00
   128091.00     273.08     117.28    0.8500       1.00
   128091.00    2432.41      98.68    0.8140       1.00
   128091.00    3920.06      79.98    0.7620       1.00
     1.046924 -3.512081
     62016.00                90.88    0.8440        1.00
     62016.00                90.88    0.8440        1.00
     62016.00                90.88    0.8440        1.00
     62016.00                93.60    0.7870        1.00
     62016.00                93.60    0.7870        1.00
    62016.00     588.77      33.93    0.4520       1.00
    62016.00    1790.69      80.09    0.5400       1.00
    62016.00     814.44      74.55    0.5210       1.00
    62016.00     111.15      76.88    0.8005       1.00
    62016.00     182.80      39.62    0.7065       1.00
    62016.00    1141.55 [illegible].21    0.7480       1.00
    62016.00     193.14      90.99    0.7880       1.00
    62016.00     117.34      92.45    0.7345       1.00
    62016.00     122.13      92.75    0.7635       1.00
    62016.00     151.03      45.64    0.8580       1.00
    62016.00    1461.38      54.05    0.8295       1.00
    62016.00    1176.97      95.06    0.9090       1.00
    62016.00     319.97      83.07    0.7530       1.00
    62016.00     121.10      91.89    0.8105       1.00
    62016.00     171.91      49.49    0.6805       1.00
    62016.00    6207.46      53.78    0.5190       1.00
    62016.00    6568.69      50.78    0.3375       1.00
    62016.00    5871.14      61.51    0.3970       1.00
    62016.00    1172.33      48.10    0.3240       1.00
    62016.00     582.00      37.27    0.3840       1.00
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 1.003495 -3.329786
 63686.00                  97.01    0.8530     1.00
 63686.00                  97.01    0.8530     1.00
 63686.00                  97.01    0.8530     1.00
 63686.00                 100.92    0.7730     1.00
 63686.00                 100.92    0.7730     1.00
63686.00     64.46        33.20    0.4570     1.00
63686.00    191.31        89.29    0.6615     1.00
63686.00    444.83        84.81    0.5275     1.00
63686.00    193.24        93.80    0.7690     1.00
63686.00     95.74        42.22    0.6670     1.00
63686.00    130.17        51.89    0.7690     1.00
63686.00    840.37       104.41    0.7395     1.00
63686.00    281.70       121.07    0.7785     1.00
63686.00     93.57       102.12    0.8100     1.00
63686.00    101.39        44.25    0.7435     1.00
63686.00    166.70        64.40    0.7065     1.00
63686.00    690.68       112.74    0.8665     1.00
63686.00    521.33       104.72    0.8155     1.00
63686.00    146.58       109.41    0.8020     1.00
63686.00    109.77        56.01    0.6695     1.00
63686.00    553.61        50.75    0.4220     1.00
63686.00    584.10        54.73    0.3345     1.00
63686.00    932.11        59.06    0.3405     1.00
63686.00   1230.05        50.47    0.2980     1.00
63686.00    526.88        41.36    0.3170     1.00
 0.993589 -3.482027
 62367.00                  68.38    0.4530     1.00
 62367.00                  68.38    0.4530     1.00
 62367.00                  68.38    0.4530     1.00
 62367.00                  73.06    0.3220     1.00
 62367.00                  73.06    0.3220     1.00
62367.00     36.54        23.31    0.2780     1.00
62367.00     79.51        75.36    0.4565     1.00
62367.00    281.96        65.62    0.3520     1.00
62367.00    167.03        78.07    0.4650     1.00
62367.00     60.93        25.41    0.3970     1.00
62367.00     42.98        35.75    0.3710     1.00
62367.00    284.47        89.69    0.4545     1.00
62367.00    189.74        92.73    0.3825     1.00
62367.00     92.43        86.71    0.4805     1.00
62367.00     64.06        31.37    0.4645     1.00
62367.00     80.54        43.37    0.4365     1.00
62367.00    261.73        97.92    0.5200     1.00
62367.00    542.09        83.00    0.4160     1.00
62367.00    127.47        93.92    0.4790     1.00
62367.00     96.72        40.79    0.3870     1.00
62367.00    237.39        49.90    0.4550     1.00
62367.00    257.80        57.97    0.3310     1.00
62367.00    413.96        56.57    0.2205     1.00
62367.00   1042.03        50.18    0.1615     1.00
62367.00    391.59        35.03    0.1650     1.00
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DEFINITION OF VARIABLES:

     B(P) - SLOPE OF STANDARD PLOT OF RELATIVE AREA VERSUS CONCENTRATION
     AP - INTERCEPT OF STANDARD PLOT OF RELATIVE AREA VERSUS CONCENTRATION
     SO - INITIAL SPECIFIC ACTIVITY, DPM/NM, OF 3H SUBSTRATE ADDED
     CO - INITIAL NUMBER OF 3H-SUBSTRATE COUNTS ADDED TO SAMPLE
     C1 - INITIAL NUMBER OF 14C DPM ADDED TO SAMPLE AS X
     C2 - NUMBER OF 3H DPM RECOVERED FROM SAMPLE AS X
     C3 - NUMBER OF 14C DPM RECOVERED FROM SAMPLE AS X
     A1 - RELATIVE AREA OF PEAK FROM COMPONENT X IN ALIQUOT OF SAMPLE
     V - VOLUME INJECTED FOR MEASUREMENT
     CE - EFFICIENCY OF CAPILLARY CAPTURE FOR THIS COMPONENT


B=  1.046924   SO=1898380.00   CO=1846948.00   AP= -3.512081   CE=0.884790


NONENZYMATIC SUBSTRATE DATA


    C1            C3           A1          V
  62016.00       90.88       0.8440       1.00
  62016.00       90.88       0.8440       1.00
  62016.00       90.88       0.8440       1.00
  62016.00       93.60       0.7870       1.00
  62016.00       93.60       0.7870       1.00


ENZYMATIC SUBSTRATE DATA


    C1            C3           A1          V
  62016.00       33.93       0.4520       1.00
  62016.00       80.09       0.5480       1.00
  62016.00       74.55       0.5210       1.00
  62016.00       76.88       0.8005       1.00
  62016.00       39.62       0.6965       1.00
  62016.00       51.21       0.7480       1.00
  62016.00       90.99       0.7880       1.00
  62016.00       92.45       0.7345       1.00
  62016.00       92.75       0.7635       1.00
  62016.00       45.64       0.8580       1.00
  62016.00       54.05       0.8295       1.00
  62016.00       95.06       0.9090       1.00
  62016.00       83.07       0.7530       1.00
  62016.00       91.89       0.8105       1.00
  62016.00       49.49       0.6805       1.00
  62016.00       53.78       0.5190       1.00
  62016.00       50.78       0.3375       1.00
  62016.00       61.51       0.3970       1.00
  62016.00       48.10       0.3240       1.00
  62016.00       37.27       0.3840       1.00
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```
BP= 1.005030     AP= -3.375531     CE= 0.685828

NONENZYMATIC PRODUCT 1 DATA

   C1            C3          A1           V
 61734.00       76.27      0.8280       1.00
 61734.00       76.27      0.8280       1.00
 61734.00       76.27      0.8280       1.00
 61734.00       79.78      0.7940       1.00
 61734.00       79.78      0.7940       1.00

ENZYMATIC PRODUCT 1 DATA
   C1            C2          C3           A1           V
 61734.00       14.52       22.86       0.4105        1.00
 61734.00       73.67       75.35       0.4670        1.00
 61734.00       94.48       64.60       0.4235        1.00
 61734.00      151.77       80.27       0.7795        1.00
 61734.00      144.60       26.40       0.5770        1.00
 61734.00       18.45       32.73       0.6320        1.00
 61734.00      495.79       87.27       0.7210        1.00
 61734.00      224.79       72.11       0.6375        1.00
 61734.00      435.25       83.53       0.7075        1.00
 61734.00      236.25       29.53       0.7395        1.00
 61734.00       41.51       50.51       0.7130        1.00
 61734.00      155.66       96.96       0.7325        1.00
 61734.00       83.03       78.33       0.7210        1.00
 61734.00      270.20       88.96       0.8045        1.00
 61734.00      249.77       42.66       0.5830        1.00
 61734.00      153.70       37.56       0.4960        1.00
 61734.00      194.15       50.26       0.3860        1.00
 61734.00      361.49       53.23       0.4340        1.00
 61734.00      631.14       43.67       0.3850        1.00
 61734.00      531.94       30.51       0.3315        1.00
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BP=  0.988246      AP= -3.357901      CE=  0.589727

NONENZYMATIC PRODUCT 2 DATA


    C1            C3          A1          V
 128091.00      163.41     1.8700       1.00
 128091.00      163.41     1.8700       1.00
 128091.00      163.41     1.8700       1.00
 128091.00      163.38     1.6890       1.00
 128091.00      163.38     1.6890       1.00


ENZYMATIC PRODUCT 2 DATA
    C1           C2          C3          A1          V
 128091.00      35.54       54.86      0.9385       1.00
 128091.00      58.90      150.90      1.2230       1.00
 128091.00     158.72      127.57      1.0495       1.00
 128091.00    1396.45      160.67      1.5425       1.00
 128091.00     635.75       60.34      1.2985       1.00
 128091.00      42.07       65.77      1.3160       1.00
 128091.00     274.97      173.57      1.4615       1.00
 128091.00    1445.56      184.33      1.6740       1.00
 128091.00    1334.55      169.50      1.5400       1.00
 128091.00     655.33       70.31      1.5250       1.00
 128091.00      48.09       93.40      1.2285       1.00
 128091.00     131.50      182.33      1.5965       1.00
 128091.00     453.02      169.02      1.6350       1.00
 128091.00    1508.75      183.81      1.6060       1.00
 128091.00     876.23       95.52      1.3680       1.00
 128091.00     268.61       99.22      1.1020       1.00
 128091.00     208.53      109.81      0.8220       1.00
 128091.00     273.08      117.28      0.8500       1.00
 128091.00    2432.41       98.68      0.8140       1.00
 128091.00    3920.06       79.98      0.7620       1.00
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BP=  1.046924      AP= -3.512081      CE=  0.884791

NONENZYMATIC PRODUCT 3 DATA

    C1            C3          A1          V
  62016.00       90.88      0.8440      1.00
  62016.00       90.88      0.8440      1.00
  62016.00       90.88      0.8440      1.00
  62016.00       93.60      0.7870      1.00
  62016.00       93.60      0.7870      1.00

ENZYMATIC PRODUCT 3 DATA
    C1           C2          C3          A1          V
  62016.00     588.77      33.93      0.4520      1.00
  62016.00    1790.69      80.09      0.5480      1.00
  62016.00     814.44      74.55      0.5210      1.00
  62016.00     111.15      76.88      0.8005      1.00
  62016.00     182.80      39.62      0.6965      1.00
  62016.00    1141.55      51.21      0.7480      1.00
  62016.00     193.14      90.99      0.7880      1.00
  62016.00     117.34      92.45      0.7345      1.00
  62016.00     122.13      92.75      0.7635      1.00
  62016.00     151.03      45.64      0.8580      1.00
  62016.00    1461.38      54.05      0.8295      1.00
  62016.00    1176.97      95.06      0.9090      1.00
  62016.00     319.97      83.07      0.7530      1.00
  62016.00     121.10      91.89      0.8105      1.00
  62016.00     171.91      49.49      0.6805      1.00
  62016.00    6207.46      53.78      0.5190      1.00
  62016.00    6568.69      50.78      0.3375      1.00
  62016.00    5871.14      61.51      0.3970      1.00
  62016.00    1172.33      48.10      0.3240      1.00
  62016.00     562.00      37.27      0.3840      1.00
```

```
BP=  1.003495      AP= -3.329786      CE=  0.780580

NONENZYMATIC PRODUCT 4 DATA

       C1            C3            A1           V
    63686.00        97.01        0.8530        1.00
    63686.00        97.01        0.8530        1.00
    63686.00        97.01        0.8530        1.00
    63686.00       100.92        0.7730        1.00
    63686.00       100.92        0.7730        1.00

ENZYMATIC PRODUCT 4 DATA
      C1             C2            C3           A1            V
    63686.00        64.46         33.20       0.4570         1.00
    63686.00       191.31         89.29       0.6615         1.00
    63686.00       444.83         84.81       0.5275         1.00
    63686.00       193.24         93.80       0.7690         1.00
    63686.00        95.74         42.22       0.6670         1.00
    63686.00       130.17         51.89       0.7690         1.00
    63686.00       840.37        104.41       0.7395         1.00
    63686.00       281.70        121.07       0.7785         1.00
    63686.00        93.57        102.12       0.8100         1.00
    63686.00       101.39         44.25       0.7435         1.00
    63686.00       166.70         64.40       0.7065         1.00
    63686.00       690.68        112.74       0.8665         1.00
    63686.00       521.33        104.72       0.8155         1.00
    63686.00       146.58        109.41       0.8020         1.00
    63686.00       109.77         56.01       0.6695         1.00
    63686.00       553.61         50.75       0.4220         1.00
    63686.00       584.10         54.73       0.3345         1.00
    63686.00       932.11         59.06       0.3405         1.00
    63686.00      1230.05         50.47       0.2980         1.00
    63686.00       526.88         41.36       0.3170         1.00
```

```
BP=  0.993589     AP= -3.482027     CE=  0.491455

NONENZYMATIC PRODUCT 5 DATA

   C1              C3            A1            V
 62367.00        68.38        0.4530        1.00
 62367.00        68.38        0.4530        1.00
 62367.00        68.38        0.4530        1.00
 62367.00        73.06        0.3220        1.00
 62367.00        73.06        0.3220        1.00

ENZYMATIC PRODUCT 5 DATA
   C1              C2             C3             A1             V
 62367.00        36.54          23.31        0.2780          1.00
 62367.00        79.51          75.36        0.4565          1.00
 62367.00       281.96          65.62        0.3520          1.00
 62367.00       167.03          78.07        0.4650          1.00
 62367.00        60.93          25.41        0.3970          1.00
 62367.00        42.98          35.75        0.3710          1.00
 62367.00       284.47          89.69        0.4545          1.00
 62367.00       189.74          92.73        0.3825          1.00
 62367.00        92.43          86.71        0.4805          1.00
 62367.00        64.06          31.37        0.4645          1.00
 62367.00        80.54          43.37        0.4365          1.00
 62367.00       261.73          97.92        0.5200          1.00
 62367.00       542.09          83.00        0.4160          1.00
 62367.00       127.47          93.92        0.4790          1.00
 62367.00        96.72          40.79        0.3870          1.00
 62367.00       237.39          49.90        0.4550          1.00
 62367.00       257.80          57.97        0.3310          1.00
 62367.00       413.96          56.57        0.2205          1.00
 62367.00      1042.03          50.18        0.1615          1.00
 62367.00       391.59          35.03        0.1650          1.00
```

EQUATIONS:

1 - 3H PRODUCT FORMED
2 - PRODUCT FORMED FROM 3H AND ENDOGENOUS SUBSTRATE
3 - PRODUCT FORMED FROM ALL SOURCES
X - ENDOGENOUS SUBSTRATE
XI - ENDOGENOUS PRODUCT
XII - NET CONVERSION OF SUBSTRATE
XIII - NET FORMATION OF PRODUCT
XIV - PRODUCT FORMED FROM ENDOGENOUS SUBSTRATE
XV - PRODUCT FORMED FROM SUBSTRATE PRECURSORS
XVI - PRODUCT FORMED FROM OTHER SUBSTRATES

ANIMAL TREATMENTS:

1(I) - CONTROL, SESAME OIL INJECTED
2(II) - ESTRADIOL INJECTED
3(III) - TESTOSTERONE INJECTED
4(IV) - DDT INJECTED

NMOLES SUBSTRATE: NONENZYMATIC INCUBATION SAMPLE

0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
302.5964	302.5964	302.5964	274.8206	274.8206

NMOLES/MG-PROTEIN, SUBSTRATE: ENZYMATIC INCUBATION

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	48.6618	24.7792	25.3664	37.0729	62.9832
2	61.4888	36.3720	33.4725	34.6214	78.6538
3	64.9663	40.3137	38.5391	37.3770	58.7261
4	2.1821	1.5321	1.4770	1.5556	2.3614

EQUATION X

1	15.6741
2	22.6047
3	25.6834
4	-7.1481

EQUATION XII

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	0.0	23.8826	23.2954	11.5889	-14.3214
2	0.0	25.1168	28.0163	26.8674	-17.1650
3	0.0	24.6527	26.4272	27.5894	6.2402
4	0.0	0.6500	0.7051	0.6265	-0.1793

```
                    PRODUCT 1

NMOLES PRODUCT: NONENZYMATIC INCUBATION SAMPLE

         0 MIN     5 MIN     10 MIN    30 MIN    60 MIN
        280.0796  280.0796  280.0796  256.8162  256.8162

NMOLES/MG-PROTEIN, PRODUCT: ENZYMATIC INCUBATION

         0 MIN     5 MIN     10 MIN    30 MIN    60 MIN
1        50.6822   17.4812   18.5000   27.3204   61.5816
2        64.1021   27.4085   29.3472   28.1022   83.0680
3        47.3132   25.3178   30.8498   30.2928   45.8517
4         2.3192    1.3505    1.4329    1.5503    1.9121

EQUATION 1

         0 MIN     5 MIN     10 MIN    30 MIN    60 MIN
1        0.0023    0.0035    0.0052    0.0067    0.0194
2        0.0024    0.0237    0.0130    0.0218    0.0334
3        0.0035    0.0068    0.0045    0.0128    0.0247
4        0.0045    0.0043    0.0075    0.0160    0.0192

EQUATION 2

         0 MIN     5 MIN     10 MIN    30 MIN    60 MIN
1        0.3350    0.5157    0.7714    1.1886    3.4433
2        0.4283    4.3161    2.3683    4.5804    7.0326
3        0.7090    1.3850    0.9144    2.9863    5.7566
4       -1.1022   -1.0404   -1.8291   -3.4371   -4.1464

EQUATION 3

         0 MIN     5 MIN     10 MIN    30 MIN    60 MIN
1        0.3350   -0.2648   -0.3674    0.4562    6.0653
2        0.4283   -0.4533   -0.5508   -0.0990   11.6227
3        0.7090    0.0621   -0.0219    0.1854    4.5354
4       -1.1022   -1.1244   -1.9892   -3.7397   -4.0419

EQUATION XI
1        20.1492
2        28.1114
3        10.9534
4        -7.1895
```

EQUATION XIII

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	0.0	-33.2010	-32.1822	-23.3618	10.8994
2	0.0	-36.6936	-34.7549	-35.9999	18.9660
3	0.0	-21.9954	-16.4634	-17.0205	-1.4615
4	0.0	-0.9687	-0.8863	-0.7689	-0.4072

EQUATION XIV

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	-50.3472	-50.1665	-49.9108	-49.4936	-47.2389
2	-63.6738	-59.7859	-61.7337	-59.5217	-57.0695
3	-46.6043	-45.9283	-46.3988	-44.3269	-41.5566
4	-3.4214	-3.3597	-4.1483	-5.7563	-6.4656

EQUATION XV

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	0.0	-0.7805	-1.1388	-0.7324	2.6219
2	0.0	-4.7694	-2.9192	-4.6794	4.5901
3	0.0	-1.3229	-0.9363	-2.8009	-1.2212
4	0.0	-0.0839	-0.1600	-0.3026	0.1045

EQUATION XVI

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	-0.3350	-32.9362	-31.8148	-23.8180	4.8341
2	-0.4283	-36.2403	-34.2040	-35.9009	7.3433
3	-0.7090	-22.0575	-16.4415	-17.2059	-5.9969
4	1.1022	0.1556	1.1028	2.9708	3.6348

```
                     PRODUCT 2

NMOLES PRODUCT: NONENZYMATIC INCUBATION SAMPLE

       0 MIN      5 MIN     10 MIN     30 MIN     60 MIN
    586.0391   586.0391   586.0391   528.7720   528.7720

NMOLES/MG-PROTEIN, PRODUCT: ENZYMATIC INCUBATION

       0 MIN      5 MIN     10 MIN     30 MIN     60 MIN
1    94.7259    45.0190    45.6145    53.4746   119.6202
2   131.1253    55.2489    59.6842    59.6514   142.3873
3    87.0086    58.1029    64.2080    57.9821    94.8595
4     3.8378     2.5776     2.4966     2.8401     3.2777

EQUATION 1

       0 MIN      5 MIN     10 MIN     30 MIN     60 MIN
1     0.0048     0.0029     0.0092     0.0639     0.0775
2     0.0055     0.0137     0.0680     0.0683     0.0808
3     0.0045     0.0063     0.0235     0.0719     0.0804
4     0.0062     0.0044     0.0053     0.0565     0.1123

EQUATION 2

       0 MIN      5 MIN     10 MIN     30 MIN     60 MIN
1     0.7090     0.4272     1.3616    11.3371    13.7433
2     1.0083     2.4973    12.3622    14.3604    16.9998
3     0.9216     1.2910     4.7976    16.7451    18.7139
4    -1.5129    -1.0613    -1.3012   -12.1633   -24.1854

EQUATION 3

       0 MIN      5 MIN     10 MIN     30 MIN     60 MIN
1     0.7090    -0.2193    -0.6485     4.3515    24.2081
2     1.0083    -0.2623    -2.8753    -0.3104    28.0954
3     0.9216     0.0579    -0.1148     1.0396    14.7439
4    -1.5129    -1.1469    -1.4151   -13.2343   -23.5761

EQUATION XI
1    30.8385
2    55.8183
3    10.9293
4   -16.0582
```

EQUATION XIII

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	0.0	-49.7069	-49.1115	-41.2513	24.8942
2	0.0	-75.8764	-71.4410	-71.4739	11.2620
3	0.0	-28.9057	-22.8006	-29.0265	7.8509
4	0.0	-1.2602	-1.3412	-0.9977	-0.5601

EQUATION XIV

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	-94.0170	-94.2988	-93.3643	-83.3889	-80.9826
2	-130.1169	-128.6280	-118.7630	-116.7649	-114.1255
3	-86.0870	-85.7176	-82.2110	-70.2635	-68.2947
4	-5.3508	-4.8991	-5.1391	-16.0011	-28.0233

EQUATION XV

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	0.0	-0.6465	-2.0101	-6.9855	10.4648
2	0.0	-2.7595	-15.2375	-14.6708	11.0956
3	0.0	-1.2331	-4.9124	-15.7056	-3.9700
4	0.0	-0.0856	-0.1139	-1.0710	0.6094

EQUATION XVI

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	-0.7090	-49.4876	-48.4630	-45.6028	0.6862
2	-1.0083	-75.6141	-68.5658	-71.1635	-16.8334
3	-0.9216	-28.9636	-22.6858	-30.0660	-6.8930
4	1.5129	-0.1133	0.0739	12.2365	23.0159

```
                              PRODUCT 3

NMOLES PRODUCT: NONENZYMATIC INCUBATION SAMPLE

        0 MIN     5 MIN     10 MIN    30 MIN    60 MIN
       302.5964  302.5964  302.5964  274.8206  274.8206

NMOLES/MG-PROTEIN, PRODUCT: ENZYMATIC INCUBATION

        0 MIN     5 MIN     10 MIN    30 MIN    60 MIN
1      48.6618   24.7792   25.3664   37.0729   62.9832
2      61.4888   36.3720   33.4725   34.6214   78.6538
3      64.9663   40.3137   38.5391   37.3770   58.7261
4       2.1821    1.5321    1.4770    1.5556    2.3614

EQUATION 1

        0 MIN     5 MIN     10 MIN    30 MIN    60 MIN
1       0.0618    0.0796    0.0389    0.0051    0.0164
2       0.0936    0.0089    0.0053    0.0055    0.0139
3       0.1147    0.0525    0.0163    0.0056    0.0147
4       0.1280    0.1435    0.1059    0.0270    0.0173

EQUATION 2

        0 MIN     5 MIN     10 MIN    30 MIN    60 MIN
1       9.1944   11.8468    5.7886    0.9130    2.9138
2      17.0130    1.6200    0.9687    1.1628    2.9222
3      23.4314   10.7300    3.3381    1.3017    3.4309
4     -31.2300  -34.9998  -25.8259   -5.8228   -3.7307

EQUATION 3

        0 MIN     5 MIN     10 MIN    30 MIN    60 MIN
1       9.1944   -6.0829   -2.7568    0.3505    5.1325
2      17.0130   -0.1701   -0.2253   -0.0251    4.8294
3      23.4314    0.4810   -0.0799    0.0808    2.7031
4     -31.2300  -37.8230  -28.0857   -6.3355   -3.6367

EQUATION XI
1      15.6741
2      22.6047
3      25.6834
4      -8.0911
```

EQUATION XIII

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	0.0	-23.8826	-23.2954	-11.5889	14.3214
2	0.0	-25.1168	-28.0163	-26.8674	17.1650
3	0.0	-24.6527	-26.4272	-27.5894	-6.2402
4	0.0	-0.6500	-0.7051	-0.6265	0.1793

EQUATION XIV

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	-39.4675	-36.8150	-42.8732	-47.7488	-45.7480
2	-44.4758	-59.8688	-60.5201	-60.3260	-58.5606
3	-41.5349	-54.2363	-61.6283	-63.6647	-61.5354
4	-33.4121	-37.1819	-28.0080	-8.0049	-5.9128

EQUATION XV

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	0.0	-17.9297	-8.5454	-0.5626	2.2187
2	0.0	-1.7902	-1.1940	-1.1879	1.9073
3	0.0	-10.2490	-3.4180	-1.2209	-0.7278
4	0.0	-2.8232	-2.2597	-0.5127	0.0940

EQUATION XVI

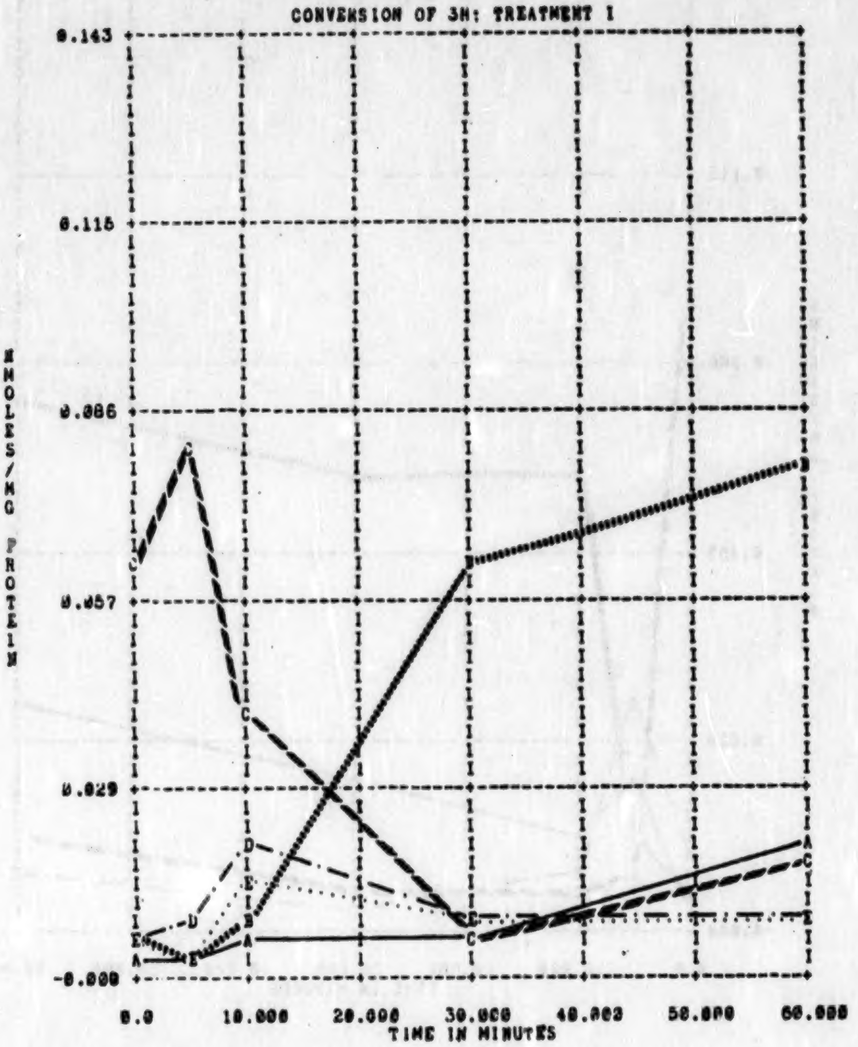
	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	-9.1944	-17.7998	-20.5386	-11.9394	9.1889
2	-17.0130	-24.9466	-27.7909	-26.8423	12.3356
3	-23.4314	-25.1337	-26.3474	-27.6702	-8.9433
4	31.2300	37.1730	27.3806	5.7090	3.8160

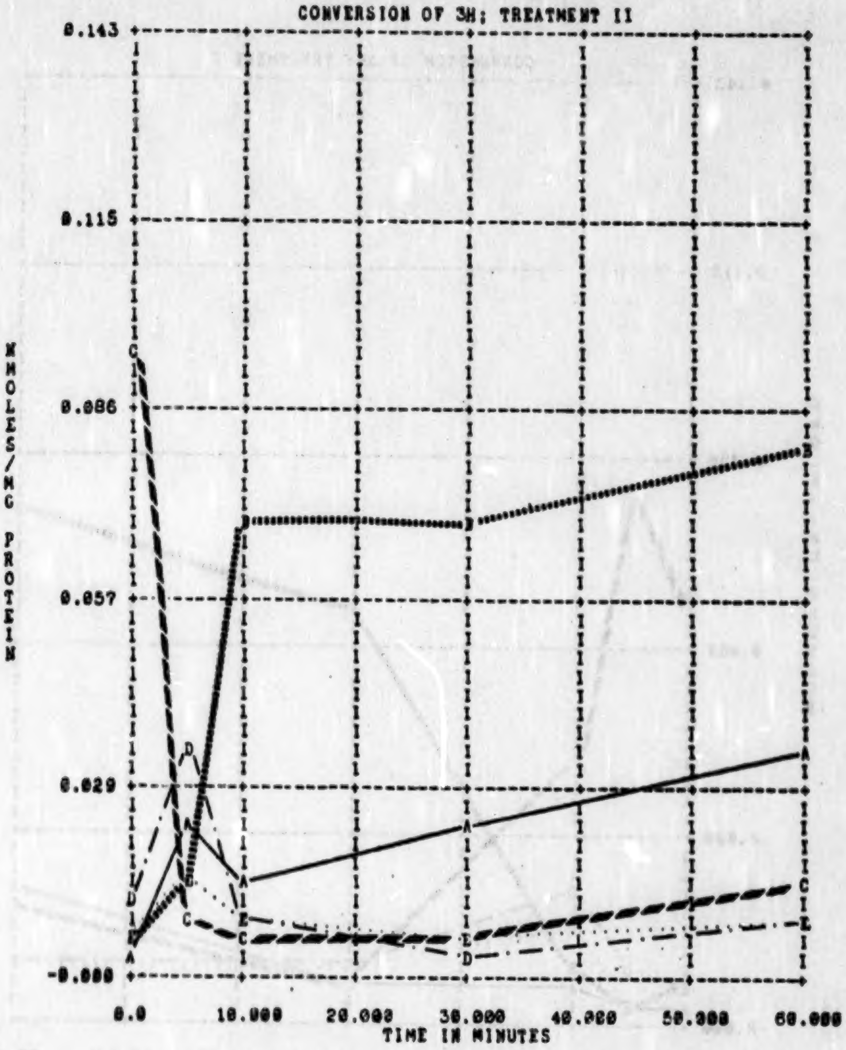
```
                    PRODUCT 4

NMOLES PRODUCT: NONENZYMATIC INCUBATION SAMPLE

       0 MIN     5 MIN     10 MIN    30 MIN    60 MIN
     242.7879  242.7879  242.7879  211.5665  211.5665

NMOLES/MG-PROTEIN, PRODUCT: ENZYMATIC INCUBATION

       0 MIN     5 MIN     10 MIN    30 MIN    60 MIN
1     41.5246   22.3201   18.7537   24.6867   47.5952
2     52.6021   25.1429   22.8225   28.1485   59.6456
3     39.3501   27.5486   27.9188   26.2812   42.8831
4      1.5628    1.1496    1.0844    1.1111    1.4419

EQUATION 1

       0 MIN     5 MIN     10 MIN    30 MIN    60 MIN
1      0.0071    0.0078    0.0192    0.0075    0.0083
2      0.0108    0.0347    0.0100    0.0039    0.0099
3      0.0113    0.0267    0.0217    0.0058    0.0085
4      0.0124    0.0122    0.0180    0.0278    0.0145

EQUATION 2

       0 MIN     5 MIN     10 MIN    30 MIN    60 MIN
1      1.0565    1.1658    2.8540    1.3361    1.4707
2      1.9661    6.3083    1.8236    0.8309    2.0778
3      2.3037    5.4522    4.4305    1.3589    1.9879
4     -3.0310   -2.9654   -4.3852   -5.9794   -3.1254

EQUATION 3

       0 MIN     5 MIN     10 MIN    30 MIN    60 MIN
1      1.0565   -0.5986   -1.3592    0.5128    2.5905
2      1.9661   -0.6625   -0.4241   -0.0180    3.4340
3      2.3037    0.2444   -0.1060    0.0844    1.5661
4     -3.0310   -3.2046   -4.7689   -6.5059   -3.0466

EQUATION XI
1      15.0569
2      21.4034
3       7.8314
4      -6.6799
```

```
EQUATION XIII

        0 MIN      5 MIN     10 MIN     30 MIN     60 MIN
1       0.0     -19.2045   -22.7709   -16.8379     6.0706
2       0.0     -27.4592   -29.7796   -24.4536     7.0435
3       0.0     -11.8014   -11.4312   -13.0689     3.5331
4       0.0      -0.4132    -0.4784    -0.4517    -0.1209


EQUATION XIV

        0 MIN      5 MIN     10 MIN     30 MIN     60 MIN
1     -40.4681   -40.3587   -38.6706   -40.1885   -40.0539
2     -50.6360   -46.2938   -50.7785   -51.7712   -50.5243
3     -37.0464   -33.8979   -34.9195   -37.9912   -37.3622
4      -4.5938    -4.5282    -5.9481    -7.5422    -4.6882


EQUATION XV

        0 MIN      5 MIN     10 MIN     30 MIN     60 MIN
1       0.0       -1.7644    -4.2131    -0.8232     1.1198
2       0.0       -6.9708    -2.2478    -0.8489     1.3562
3       0.0       -5.2078    -4.5365    -1.2745    -0.4217
4       0.0       -0.2392    -0.3837    -0.5265     0.0787


EQUATION XVI

        0 MIN      5 MIN     10 MIN     30 MIN     60 MIN
1      -1.0565   -18.6059   -21.4117   -17.3507     3.4801
2      -1.9661   -26.7966   -29.3555   -24.4356     3.6095
3      -2.3037   -12.0458   -11.3252   -13.1532     1.9669
4       3.0310     2.7914     4.2905     6.0541     2.9257
```

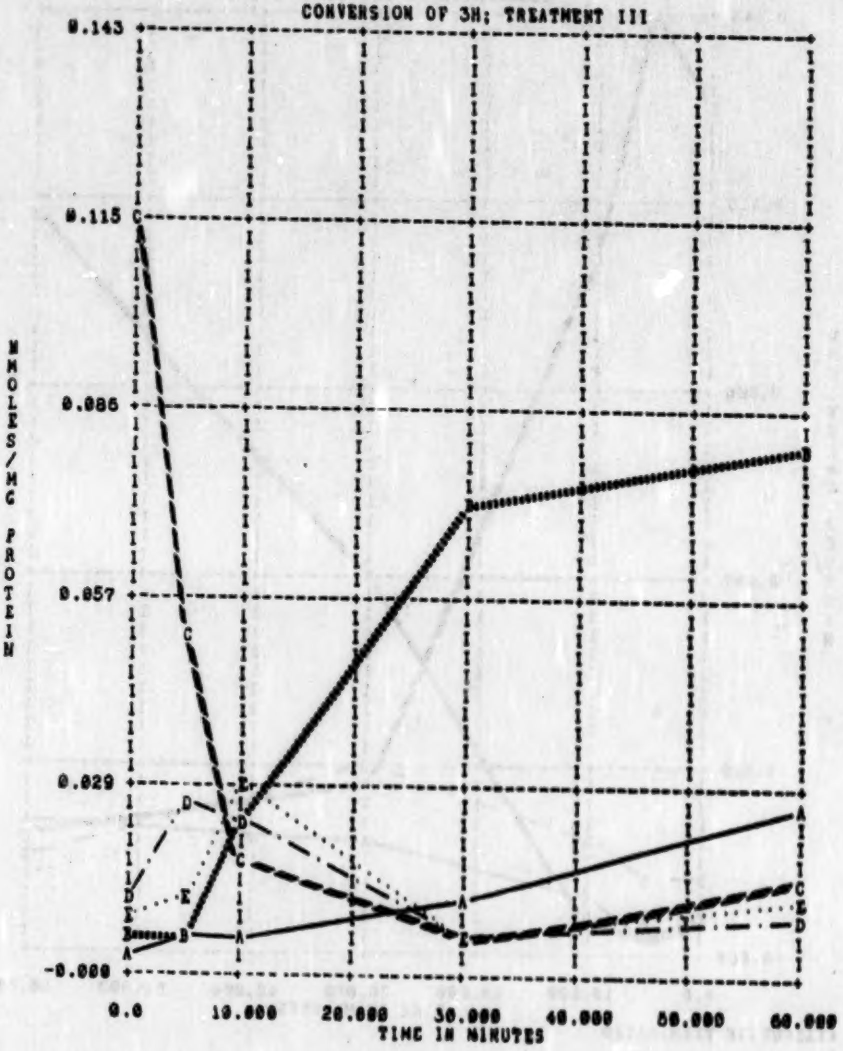
```
                         PRODUCT 8

NMOLES PRODUCT: NONENZYMATIC INCUBATION SAMPLE

      0 MIN     5 MIN     10 MIN    30 MIN    60 MIN
    173.2276  173.2276  173.2276  114.9919  114.9919


NMOLES/MG-PROTEIN, PRODUCT: ENZYMATIC INCUBATION

      0 MIN      5 MIN     10 MIN     30 MIN     60 MIN
1    33.8900    17.2686    15.2664    16.9815    44.4992
2    34.8253    17.0276    13.8450    18.6271    49.7619
3    34.1568    18.0429    17.0045    17.3189    32.1739
4     1.6190     1.0117     0.6888     0.5676     0.8309


EQUATION 1

      0 MIN      5 MIN     10 MIN     30 MIN     60 MIN
1     0.0056     0.0038     0.0154     0.0077     0.0086
2     0.0051     0.0134     0.0086     0.0045     0.0086
3     0.0079     0.0114     0.0279     0.0058     0.0101
4     0.0053     0.0050     0.0082     0.0232     0.0125


EQUATION 2

      0 MIN      5 MIN     10 MIN     30 MIN     60 MIN
1     0.8353     0.5622     2.2896     1.3588     1.5229
2     0.9227     2.4344     1.5705     0.9466     1.8135
3     1.6185     2.3295     5.6921     1.3481     2.3553
4    -1.2945    -1.2101    -1.9911    -4.9892    -2.6858


EQUATION 3

      0 MIN      5 MIN     10 MIN     30 MIN     60 MIN
1     0.8353    -0.2887    -1.0904     0.5216     2.6825
2     0.9227    -0.2557    -0.3653    -0.0205     2.9971
3     1.6185     0.1044    -0.1362     0.0837     1.8556
4    -1.2945    -1.3077    -2.1654    -5.4285    -2.6181


EQUATION XI
1        15.0054
2        12.5653
3        11.6685
4        -4.2621
```

```
EQUATION XIII

          0 MIN      5 MIN     10 MIN     30 MIN     60 MIN
1         0.0      -16.6214   -18.6236   -16.9084    10.6092
2         0.0      -17.7977   -20.9803   -16.1982    14.9366
3         0.0      -16.1139   -17.1524   -16.8380    -1.9829
4         0.0       -0.6073    -0.9301    -1.0513    -0.7881

EQUATION XIV

          0 MIN      5 MIN     10 MIN     30 MIN     60 MIN
1       -33.0547   -33.3277   -31.6003   -32.5311   -32.3671
2       -33.9025   -32.3909   -33.2548   -33.8787   -33.0118
3       -32.5383   -31.8273   -28.4647   -32.8087   -31.8016
4        -2.9134    -2.8290    -3.6101    -6.6082    -4.3048

EQUATION XV

          0 MIN      5 MIN     10 MIN     30 MIN     60 MIN
1         0.0       -0.8509    -3.3800    -0.8372     1.1596
2         0.0       -2.6900    -1.9357    -0.9671     1.1836
3         0.0       -2.2251    -5.8283    -1.2644    -0.4996
4         0.0       -0.0976    -0.1742    -0.4393     0.0677

EQUATION XVI

          0 MIN      5 MIN     10 MIN     30 MIN     60 MIN
1        -0.8353   -16.3327   -17.5331   -17.4299     7.9267
2        -0.9227   -17.5420   -20.6150   -16.1777    11.9395
3        -1.6185   -16.2184   -17.0162   -16.9216    -3.8386
4         1.2945     0.7004     1.2352     4.3771     1.8300
```

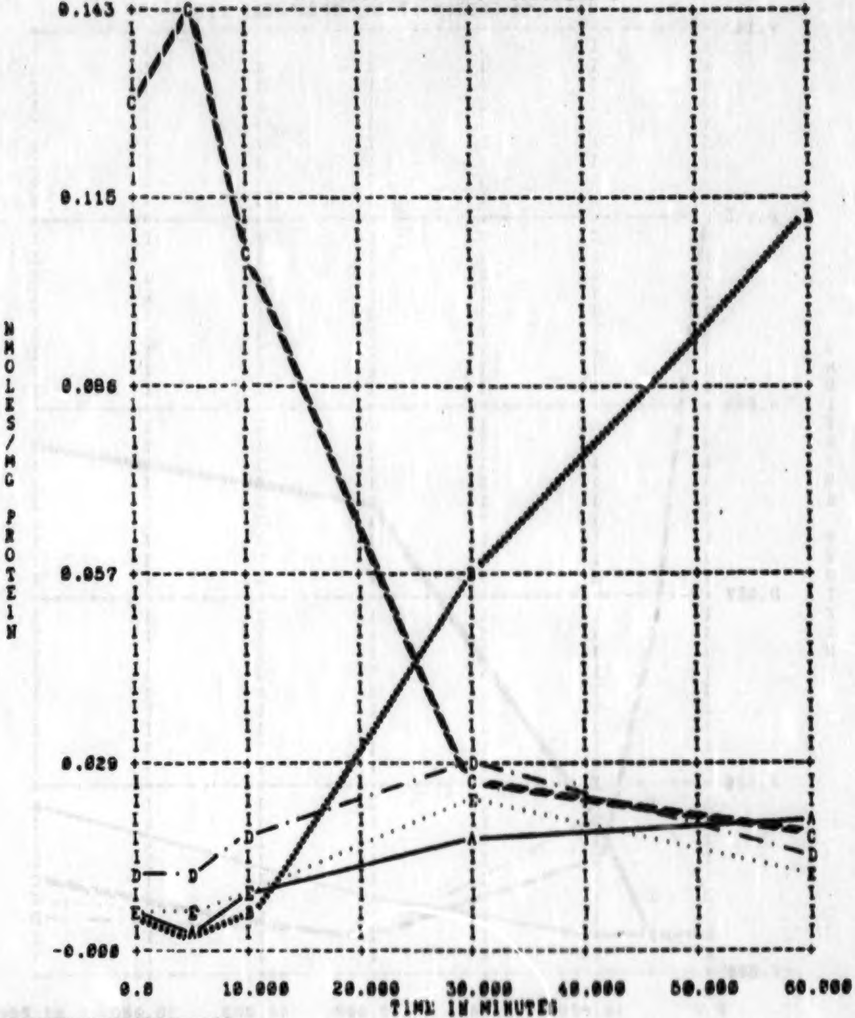

CONVERSION OF 3H: TREATMENT I
NMOLES/MG PROTEIN
0.143
0.115
0.086
0.057
0.029
-0.000
0.0
10.000
20.000
30.000
40.000
50.000
60.000
TIME IN MINUTES
A
B
C
D
E


CONVERSION OF 3H; TREATMENT II
0.143
0.115
0.086
0.057
0.029
-0.000
NMOLES/MG PROTEIN
0.0
10.000
20.000
30.000
40.000
50.000
60.000
TIME IN MINUTES
A
B
C
D
E

CONVERSION OF 3H; TREATMENT III



CONVERSION OF 3H: TREATMENT IV



*EXECUTION TERMINATED
*